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PRINCIPAL INVESTIGATOR: Jennifer Richer, Ph.D.

CONTRACTING ORGANIZATION: University of Colorado
                                                  Aurora, CO 80045

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our most significant findings over the duration of this idea award"MicroRNA-200c: A Novel Way to Attack Breast Cancer Metastases by Restoring the Epithelial Phenotype" grant are evident in our 8 publications (3 of which are reviews). The miR-200 family is the most differentially expressed miRNA in ER+ versus triple negative breast cancer (TNBC) cell lines and clinical samples. We find that miR-200 affects multiple aspects of epithelial to mesenchymal transition (EMT). Restoration of miR-200c to TNBC decreases migration and invasion and represses a whole program of nonepithelial genes normally expressed in cells of mesenchymal or neuronal origin. We made the novel observation that restoration of miR-200c enhances sensitivity to anoikis (cell death induced by detachment) and that this is mediated by direct targeting of TrkB. This year we published that one of the ligands of the TrkB receptor, NTF3 is also a target of miR-200c and the loss of miR-200cin TNBC sets up an inappropriate autocrine loop that provides a signal that allows TNBC cells to survive unattached and thereby be able to metastasize. Lastly, in as yet unpublished work we induce miR-200c in TNBC cells in the blood stream to determine if fewer cells are able to survive and form metastases in the lungs of mice.

15. SUBJECT TERMS
microRNA, EMT, invasion, ZEB1, epithelial to mesenchymal transition, anoikis

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INTRODUCTION:
In this research study we characterized the role of the microRNA, miR-200c, in breast cancer progression. We have found that breast cancer cells that retain E-cadherin (an a marker of mor normal epithelial structure) and estrogen receptors (ER) have high levels of miR-200c. In contrast, the more de-differentiated and more aggressive cell lines that have lost ER and E-cadherin have low miRNA200c. We and others have now published that miR-200c is a potent protector of the epithelial phenotype and protects against epithelial to mesenchymal transition (EMT). EMT, a process that occurs during normal development to help cells become more mobile to achieve normal developmental processes such as closure of the neural crest or the palate, is co-opted in cancer such that cells can leave the primary site and metastasize. Reintroduction of miR-200c results in restoration of the classical epithelial marker E-cadherin, by directly targeting ZEB, a repressor of E-cadherin, and we published that it also results in reduced motility and invasiveness, and increased sensitivity to chemotherapeutic drugs. We also recently published that restoration of this powerful microRNA also affects an additional understudied step in the metastatic cascade, called anoikis resistance. Normal well-differentiated epithelial cells should be attached to each other and a basement membrane, and if they sense that they are no longer attached they undergo a form of cell death (apoptosis) called anoikis (greek for homeless), which is defined as detachment induced cell death. However, carcinoma cells, particularly the highly de-differentiated triple negative subtype of breast cancer, are resistant to anoikis, whereas the more well-differentiated less aggressive ER+ subtype is still sensitive to anoikis. Our *in vitro* and *in vivo* preclinical data demonstrate that restoration of miR-200c has potential as a therapeutic strategy because it targets and represses many genes involved in multiple steps of breast cancer progression.

BODY:
In total we published 6 manuscripts that resulted from our idea award W81XWH-09-1-0124 **"**MicroRNA-200c: A Novel Way to Attack Breast Cancer Metastases by Restoring the Epithelial Phenotype" funded 01/19/2009-11/18/2012. The following summarizes our findings and how they fit with findings of others. References highlighted in bold stemmed directly from this grant. We then summarize progress in accordance with the original Statement of Work, with emphasis on the last year (Task 3) since that work was not reported in previous progress reports.

The miR-200 family (most strikingly, miR-200c) are by far the most differentially expressed miRNA in estrogen receptor alpha (ER) positive versus triple negative breast cancer (TNBC) cell lines and clinical samples. Many miRNAs are downregulated in aggressive breast cancers (1-3). We profiled luminal A breast cancer cell lines (MCF7 and T47D) which are ER+, E-cadherin+ and negative for mesenchymal markers, versus triple negative breast cancer (TNBC) lines (MDA-MB-231 and BT549) cells, which are negative for ER and E-cadherin, but positive for mesenchymal markers. The miR-200 family, including miR-200c (53 fold higher in luminal A cells) (**4**). Two-thirds of the 34 differentially expressed miRNAs are more abundant in luminal A and are lost (or present at much lower levels) in TNBC.

Originally, miR-200c was discovered to bind the 3'UTR of ZEB1/2 and cause degradation of these transcripts, turning off the ZEB1 protein production and thereby relieving transcriptional repression of E-cadherin (5). The miR-200 family shares a high degree of homology in their seed sequence, and are capable of repressing ZEB1/2, leading to re-expression of E-cadherin (6-8). Thus through our work and others, this miRNA family has been termed "guardian of the epithelial phenotype." Through the work that we have done for this grant, we have found that miR-200c is the most highly expressed in estrogen receptor alpha (ER) positive breast cancer and it represses not only ZEB1/2, but a whole program of transcripts normally expressed only in cells of mesenchymal origin **(9).** A double negative feedback loop has been demonstrated in which ZEB1 transcriptionally represses miR-200 family members (10, 11) allowing for plasticity between the epithelial and mesenchymal state (12). Supporting the idea that the miR-200 family is lost during breast cancer progression, miR-200c and miR-200a were significantly decreased in breast cancer metastases as compared to matched primary tumors (13). Further, restoration of miR-200c to breast cancer stem cells isolated from a patient sample

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abolished tumor initiating capacity in mouse mammary glands, indicating that miR-200c infected cells had lost the ability to self-renew and proliferate *in vivo* (14). Thus, some of the miR-200's ability to repress metastasis may be due to its ability to repress "stemness" via targeting of genes such as BmI1, KLF4 and Sox2 (14, 15). The miRNA profile of mammary stem cells has been demonstrated to be remarkably similar to that of TNBCs that have undergone EMT (14). Figure 1 is from a review that we authored (**16**) (paper #3 in appendix), in which we summarize our findings as well as those of others. Highlighted in red are our contributions to the body of knowledge about this critical miRNA family. Recently we (**17, 18**) described a unique way that miR-200c executes its myriad effects on breast cancer progression by protecting against a step in the metastatic cascade, anoikis resistance, which allows tumor cells to survive unattached in the bloodstream.

Figure 1. The miR-200 family directly targets genes involved in a variety of processes that contribute to tumorigenesis and metastasis. Many of the genes that miR-200c targets and represses are non-epithelial genes or genes normally expressed in stem cells or neurons or fibroblasts that get inappropriately expressed. Proteins encoded by these key targets affect multiple aspects of EMT that lead to cancer progression. The categories highlighted in red are those that the Richer lab has contributed substantially.
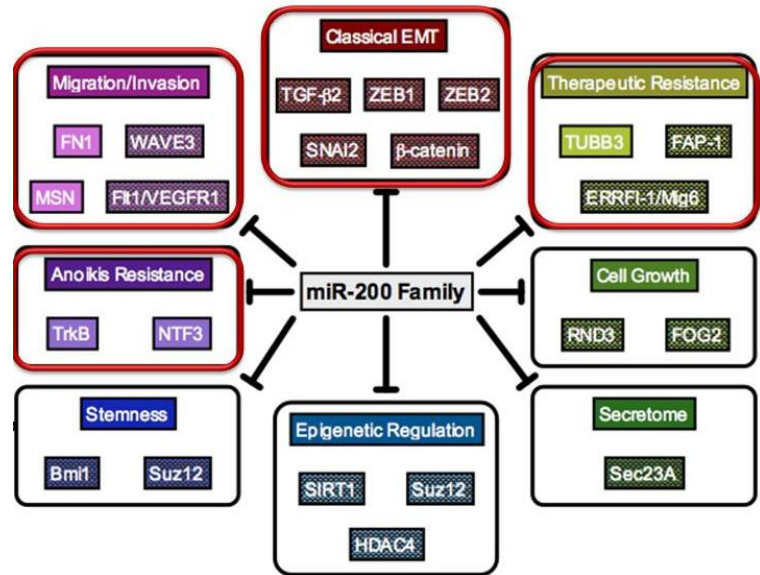
In contrast, to miR-200c, which is high in the luminal A subtype and extremely low to absent in TNBC cells, the main miRNAs that are higher in TNBC are the miR-221/222 family and by *in situ* hybridization on FFPE clinical specimens, we find that these are high in TNBC (**4**). These miRNA were known to directly target ER (19, 20), but importantly we found that they also target Dicer1 (**4**), an RNase III-type nuclease critical for the final step of miRNA biogenesis. Figure 2 summarizes the known functions of miR-221/222 including our finding that they directly target Dicer.

Figure 2. Direct targets of miR-221/222. miR-221/222 directly target and down-regulate genes associated with differentiation or tumor suppression.
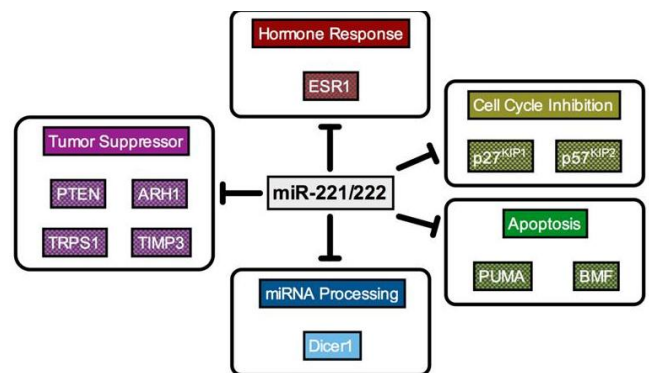
miR-200 and epithelial to mesenchymal transition (EMT)-
In summary, we have now found that restoration of miR-200c to breast, endometrial and ovarian cancer cells decreases migration and invasion. Restoration of miR-200c induces significant decreases in mRNAs targets that are normally only expressed in cells of mesenchymal or neuronal origin, but get inappropriately expressed in TNBCs that have lost miR-200c expression (**9**). We published this in the first manuscript that we put out right after obtaining this award (paper # 6 in the appendix). These targets include not only ZEB1 and 2 (transcriptional repressors of E-cadherin),resulting restoration of the epithelial hallmark E-cadherin), but also a whole program of non-epithelial genes normally expressed in cells of mesenchymal or neuronal origin We observed that other polarity associated genes such as cadherin 11 (CDH11), tight junction encoding genes *OCLN* and MARVELD2 increase when miR-200c is restored (**9**).

miR-200c and migration and invasion- miR-200c dramatically reduces migration and invasion when it is restored to various types of de-differentiated carcinoma cells. Importantly, we noted that the ability of miR-200c to reduce migration/invasion of TNBC cells does not depend on E-cadherin re-expression, since even in cells in which E-cadherin is not restored (due to methylation), migration and invasion are still dramatically reduced by restoration of miR-200c (**21**).We identified Fibronectin 1 (*FN1*) and moesin (*MSN1*) as new direct targets repressed by miR-200c, that mediate the ability of miR-200c to inhibit migration and invasion. FN1, MSN1 or both are expressed in TNBC lines that have undergone EMT and lost miR-200c, whereas they are not expressed in breast cancer cells that have high miR-200c and express epithelial markers. FN1 is a hallmark of EMT signifying a mesenchymal phenotype and the genes encoding FN1 and MSN, an actin cytoskeleton remodeling protein are both repressed when miR-200c is restored to TNBC **(18).** See later in report for more details.

miR-200c and anoikis sensitivity- Our newest contribution to this field is the novel observation that restoration of miR-200c enhances sensitivity to anoikis (detachment induced cell death) that this is mediated by direct targeting of *TrkB* (**18**) and we recently found that one of the ligands of the TrkB protein, NTF3, is also a target of miR-200c (**17**). We demonstrate that TNBC cells in suspension dramatically upregulate NFκB activity, which drives transcription of *TrkB* and *NTF3* and these transcripts are allowed to be translated if miR-200c is absent, as it is in TNBC that has undergone EMT (**17**).

miR-200c and chemosensitivity- We discovered that restoration of miR-200c greatly enhances chemosensitivity to taxanes, and direct repression of one specific target, class III beta-tubulin (TUBB3), an isoform of tubulin normally expressed in neurons, and upregulated in carcinomas resistant to microtubule targeting chemotherapeutic agents, is responsible for this attribute. We proved that introduction of exogenous, non-targetable TUBB3, lacking miR-200c target sites in the 3'UTR, could completely reverse the ability of miR-200c to restore chemosensitivity to paclitaxel (**9, 21**). This finding demonstrates that even though miRNAs are known for their ability to target many genes, it is possible for a single target to mediate an important, clinically relevant phenotype.

Statement of Work

Task 1 Aim 1 – To determine the mechanisms by which miR-200c serves to maintain the epithelial nature of breast cancer cells and how its loss leads to epithelial to mesenchymal transition (EMT).

a.   We will confirm that restoration of miR200c reduces migration and invasion capacity and renders them more sensitive to chemotherapeutic agents. These studies were performed in the first 6 months of the first year. In the first progress report, we showed that we identified 18 genes statistically significantly altered upon restoration of miR-200c, which are also putative direct targets of miR-200c. We experimentally confirmed 5 of these as direct targets using a luciferase reporter vector, pMIR-Report (18). We showed that TNBC cells that have lost miR-200c express either FN, MSN or both, while luminal A breast cancer cells do not express these proteins (figure 2a in the paper 2 in the appendix) (18). Restoration of miR-200c to TNBC cells dramatically reduces expression of these proteins and inhibits migration (see paper 2 in appendix, figures 4 and 5 in that paper).
We found that class III beta-tubulin (TUBB3) is a direct target of miR-200c and it is known to be overexpressed in many types of carcinomas that become resistant to microtubule targeting chemotherapeutic agents (taxanes) (REFS). In endometrial and ovarian cancers we find that restoration of miR-200c to tumor cells that have lost it results in a decrease of the TUBB3 protein since the gene is a direct target of miR-200c and we show that this greatly enhances sensitivity to paclitaxel by up to 85% (22). We therefore hypothesized that this would be true for breast cancers. However, although overexpression of TUBB3 is reported clinically in primary breast cancers that are resistant to microtubule targeting chemotherapeutic agents and is known to be one of 3 major causes of resistance to this type of chemotherapy in breast cancer (23-27), we were having a difficult time finding a cell

line that was resistant to paclitaxel because of high TUBB3. We reported our efforts to do so in our year 2 progress report. We also showed in the year 2 progress report that we figured out a way to see if TUBB3 was high even in the presence of miR-200c in some cases because of the TUBB3 3'UTR getting truncated and we would like to utilize that on clinical samples in the future We did find two lines recently isolated from a patient, by Dorraya El-Ashrey and Mark Lippman at University of Miami, that have extremely low miR-200c and high TUBB3 and is very resistant to paclitaxel. In the future we hope to proceed to putting miR-200c into that line and determining if we can reverse chemoresistance. We also now have approval to place primary breast tumors that are chemoresistant directly into nod-scid gamma mice to expand them and many of these tumors do have high TUBB3 so we are determining if we can get them to take up miR-200c and if this will enhance their sensitivity to paclitaxel. We have tried restoring miR-200c to the BT549 cells both stably and inducibly, but they are fairly sensitive to paclitaxel already, so inducing miR-200c does not make them more sensitive even though it does slightly reduce TUBB3 levels.

b.   To determine other mechanisms by which miR-200c serves to maintain the epithelial nature of breast cancer cells.

MiR-200c restores sensitivity to anoikis- an important attribute of epithelial cells. We identify anoikis resistance as another aspect of loss of epithelial differentiation. Anoikis, or death by detachment, is a property displayed by epithelial cells. Epithelial cells undergo anoikis, or apoptosis in response to lack of adhesion. Very aggressive breast cancers that have undergone epithelial to mesenchymal transition, often exhibit anoikis resistance, which is necessary for them to survive in the vasculature and lymphatics. We have found that restoration of miR-200c reverses anoikis resistance (Figure8 in 2nd paper in appendix) (18). Results of these experiments indicate that if NTRK2 cannot be repressed by miR-200c, it can no longer enhance anoikis sensitivity (figure 9 in paper 2 in appendix). These results are very significant in that they are the first to demonstrate that restoration of miR-200c to triple negative breast cancer can restore sensitivity to anoikis and therefore perhaps cause cancer cells to no longer be viable in the bloodstream when they are unattached to each other or basement membrane. Further, prove that NTRK2 is the target is responsible for this property of miR-200c. we recently found that one of the ligands of the TrkB protein, NTF3, is also a target of miR-200c (17). We demonstrate that TNBC cells in suspension dramatically upregulate NFκB activity, which drives transcription of *TrkB* and *NTF3* and these transcripts are allowed to be translated if miR-200c is absent, as it is in TNBC that has undergone EMT (17) (Paper #1 in the appendix). Later under progress for Task 3 we perform an experiment to put the TNBC cells that we created with inducible miR-200c back in to mice in the bloodstream to determine if inducing miR-200c will affect anoikis sensitivity *in vivo* (ability of the cells to survive in the bloodstream to proceed to establish metastases).

Apical basal polarity -another aspect of "epithelialness". To determine the extent of the ability of miR-200c to restore polarity to aggressive breast cancer cell lines we performed 3-D culture in Matrigel following restoration of miR-200c. MDA-231and BT549 were transfected with pLNCX2-ZsGreen and pLNCX2-DsRed-Express (Clontech) and selected with neomycin. Following selection DsRed expressing cells were transfected with 50 nM scrambled negative control (Ambion) and ZsGreen expressing cells were transfected with 50 nM miR-200c mimic (Ambion). Cells were then plated in growth factor reduced (GFR) Matrigel (BD Bioscience) in 8 well chamber slides using the 3-D on top method. Briefly, the bottom of the each well was coated with GFR Matrigel, cells were added and allowed to adhere before replacement of medium with medium supplemented with 10% GFP Matrigel. Cells were plated either negative control alone (neg), miR-200c alone (200c) or mixed negative control:miR-200c (1:1) (mixed) and allowed to grow for 7 days. Images were acquired at day1, day 3 and day 7 and quantitated to determine size and circularity of colonies. We found that on day 7 at the conclusion of the experiment with the MDA-MB-231 cells, miR-200c colonies are 71.5% smaller and 87.6% more circular mixed colonies are 60.7% smaller and 60.1% more circular

Unpublished preliminary data: Restoration of miR-200c to MDA-MB-231 and BT-549 TNBC cells, which typically form "stellate" structures in 3D culture, causes TNBC cells to form small rounded balls reminiscent of ER+ luminal A cells (Fig 3).

Figure 3. miR-200c decreases size and increases circularity of TNBC MDA-231 and BT549 cells in 3-D culture. MDA-231 (left) and BT549 (right) cells stably expressing DsRed or ZsGreen were transfected with a scrambled negative control or miR-200c mimic, respectively, and plated in GFR Matrigel. 10 representative images were taken for each condition negative control (neg), miR-200c (200c) or colonies containing negative and miR-200c cells (mixed) at each time point. A. Representative images for each condition at each time point. B. (Top) The cross-sectional area was determined for each colony. (Bottom) Metric showing how closely the colony approximates a circle with 1 being a perfect circle. Points, each colony, lines, mean colony size. ANOVA.
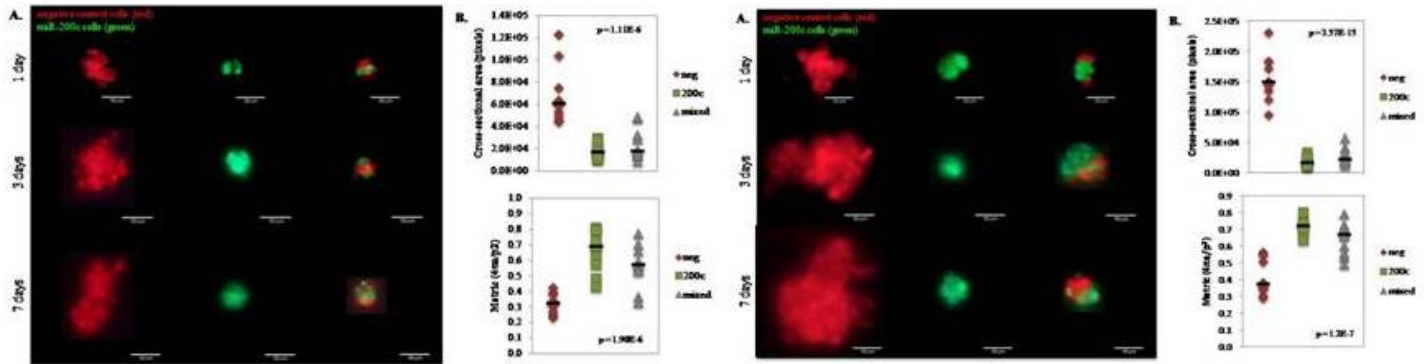
The implications of these studies are that not only can miR-200c cause TNBC cells in 3-D culture in Matrigel to slow their proliferation and form more rounded colonies, but they seem to confer this property on adjacent TNBC cells that have not had miR-200c restored. In the past year we have discovered that while miR-200c renders TNBC MDA-MB-231 colonies rounder and smaller, while inhibition of miR-222 activity alone makes the colonies rounder, but not smaller.

Progress: In an as of yet unpublished finding, we find that simultaneous restoration of miR-200c and inhibition of miR-222 activity appears to be inducing hollowing out of the structures (Fig 4). We now need to confirm whether miR-200c can reverse anoikis resistance of TNBC cells in 3D culture by staining for caspase 3 and confocal microscopy. We therefore have not published these results yet, but hope to in the next year.
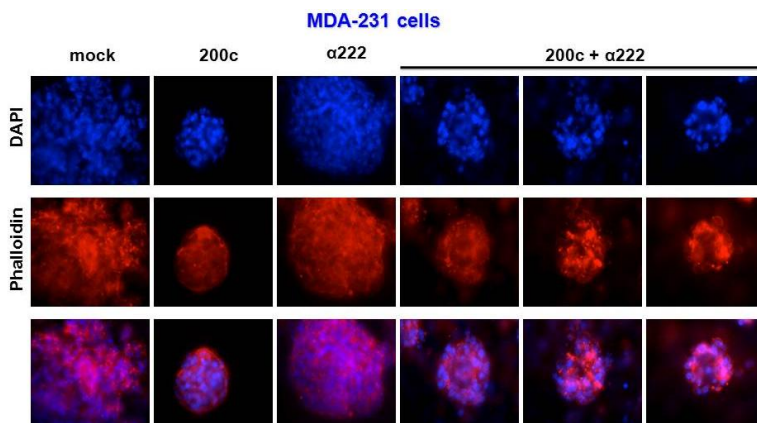

Figure 4. Effects of restoration of miR-200c and inhibition of miR-222 on MDA-231 cells in 3D culture. MDA-231 cells were mock transfected or transfected with 50 µM miR-200c mimic, 50 µM miR-222 antagomiR or 50 µM miR-200c mimic plus 50 µM miR-222 antagomiR. Cells were then plated in growth factor reduced Matrigel and allowed to grow for 7 days at which time they were fixed and stained with phalloidin (to show actin localization and thus cell structure) and DAPI (to show nuclei).

Task 2. Aim 2 – To examine miR-200c in normal breast and clinical specimens of breast cancers with varying metastatic potential and determine the mechanisms whereby miR-200c is lost or suppressed in aggressive breast cancers.

a.   To examine miR-200c in normal breast and clinical specimens of breast cancers with varying metastatic potential. Progress: We have developed the protocol for *in situ* hybridization in clinical specimens. We have had to work out this protocol with a different miRNA, miR-222 that targets the estrogen receptor and Dicer, because the LNA probe for miR-200c is not performing. The results from the miR-222 in

situ published in paper #4 in the appendix figure 3 (4). We obtained IRB approval to perform the in situ hybridization on 30 luminal A breast cancers as compared to 30 TNBC.

Progress this year. Unfortunately even though the company (Exiqon) that is the only company that makes these probes for in situ hybridization for miRNAs redesigned the probe for miR-200c, the new one does not work either. By not work I mean that it doesn't stain even breast cancer cell lines that we know by RT-PCR are high for miR-200c or the cells in which we restore it with the inducible lentiviral system. We consequently have not shown yet that it is turned off in TNBC clinical samples that have undergone EMT. However because of a particular paper it is pretty well accepted now that in all breast cancer stem cells miR-200c is downregulated and it is also down in normal mammary stem cells (14). However, in the future it would be of interest to perform laser capture microdissection to prove by RT-PCR that miR-200c is down in TNBCs (which all have a high percentage of stem cell-like cells) compared to adjacent uninvolved epithelium.

Figure 5. In situ hybridization for miR-222 and immunohistochemistry for ESR1 in luminal and triple negative clinical *MiR-222 in situ staining with a scrambled negative control is shown at the bottom. Double-DIG LNA-modified DNA probe complementary to mature miR-222 or scramble control (Exiqon) were hybridized overnight at 50C and washed in SSC at increasing stringency (5 to 0.2X SCC) at 50°C, then with PBST at room temperature. Slides were incubated for 1h with blocking solution (TBST, 1% BSA, 0.1% FBS) and then 1h with 1:2000 dilution of anti-Digoxigenin antibody (Roche). ERα staining with antibody 1D5 (DAKO) is brown and miR-222 staining is purple. (400X magnification).*
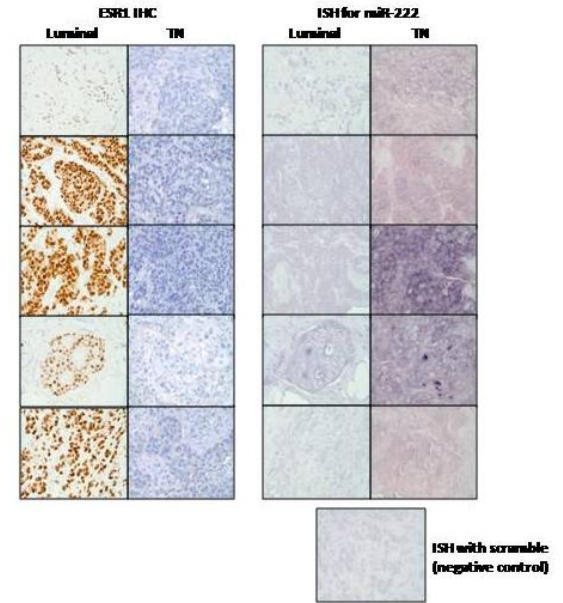
2b. Determine the mechanism whereby miR-200c is lost or suppressed in aggressive breast cancers. In the year 2 progress report I showed the progress that we had made on this aspect of the work, however, while we were in the process of optimizing the probes to make the methylation specific primers for this work it was published that the miR-200c promoter is silenced by methylation (28). Interestingly, manuscripts also reported a reciprocal relationship between miR-200c and ZEB1, whereby not only does miR-200c repress ZEB1, which is a transcription al repressor of miR-200c, but ZEB1 also represses miR-200c. We find that complete knockdown of ZEB with shRNA in TNBC can result in re-expression not only of E-cadherin, but also restoration of miR-200c by relief of transcriptional repression by ZEB1 (see MDA-MB-231 in Fig 6). However, this was not true in the BT-549 cells indicating that in some cells miR-200c expression must be either silenced by methylation or by mutation of the binding site for some postitive regulator or a mutation that prevents expression by other means, or a microdeletion in these cells. We would still like to investigate this possibility further since it may be the case in clinical specimens. However, this will involve the design of a very small FISH probe; which we have not yet done but hope to in the future. This data demonstrates that direct manipulation of miR-200c is better as a potential therapy than trying to restore it by knocking down ZEB1

Figure 6. Figure 1: Reciprocal repression between ZEB1 and miR-200c occurs in some, but not all cell types. *Western blot for ZEB1 and α-tubulin loading control, and real time RT-PCR for miR-200c in TNBC MDA- MB-231 cells (left)*

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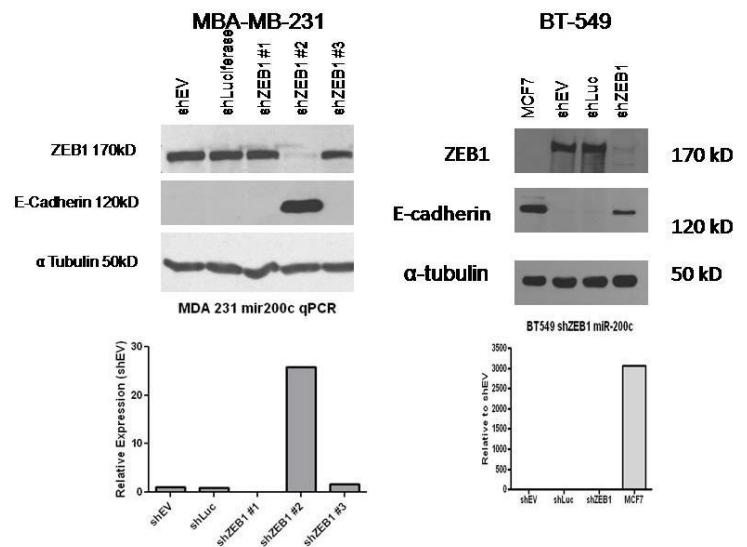
and BT549 cells (right) stably transfected with shRNA lentiviral vector targeting ZEB1 (shZEB), luciferase (shLuciferase), or empty vector (shRNA EV) and real time RT-PCR for miR-200c (bottom left and right).

Task 3. Aim 3: To determine the effects of restoration of miR-200c levels on tumor metastasis using two *in vivo* models of metastasis.

Progress: In order to study the effects of miR-200c on various steps in the metastatic cascade independent of its effects on primary tumor grow rate, it was necessary to create cells in which miR-200c can be expressed in an inducible manner. We used the pTripz system (ThermoFisher) in which the Tet-inducible promoter and the Reverse tet-transactivator rtTA3 are expressed on the same lentiviral vector and turbo RFP can be used to track inducible miRNA expression. We cloned the pri-miR-200c into the pTripz system and infected BT549 and MDA-MB-231 cells into which we had previously introduced a retroviral vector expressing luciferase (SFG-nesTGL) so that they could be used for *in vivo* IVIS imaging. We selected individual clones that 1) demonstrated no leakiness (no induction of miR-200c in the absence of doxycyclin) and 2) that induced miR-200c to levels comparable to those found in luminal A MCF7 or T47D cells. We selected a clone that is not leaky (miR-200c does not come on in absence of doxycycline) Figure 7.
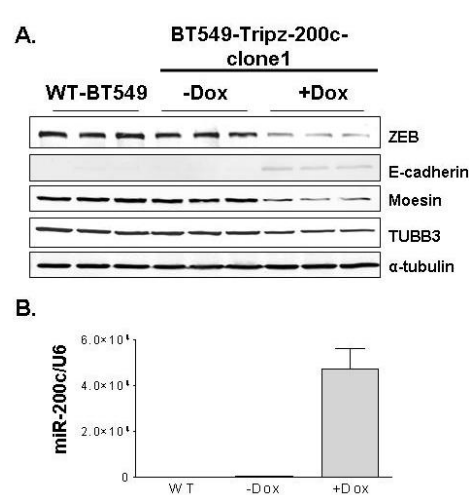
Figure 7. An inducible miR-200c lentiviral vector stably integrated into BT549 TNBC cells causes repression of ZEB1 and MSN1 protein and induces expression of E-cadherin. BT549-Tripz-200c-clone1 was cultured in triplicate in the presence or absence of 1ug/ml DOX for 96h (passage 5). A. WB for ZEB1, MSN1, and E-cadherin. B. qRT-PCR for miR-200c.

Late this year we obtained IACUC and DOD approval for our animal protocol to use these cells to address the question of whether miR-200c could affect survival of cells in vivo in the blood stream to metastasize since given what we now know about how it restores sensitivity to anoikis, we hypothesized that restoration of miR-200c should make breast cancer cells that are resisting anoikis to survive in the bloodstreatm now undergo anoikis. We had also engineered these cells to express luciferase and GFP off of a retroviral vector TGL.

As a control to make sure our method for retrieving and identifying human tumor cells in mouse blood would work, we spiked in different amounts of these breast cancer cells into mouse blood and performed the flow cytometric analysis before going into animals (Fig 8)

- Test run – spike in known numbers of cells into whole blood, go through lysis and staining procedure, see if cells are detectable

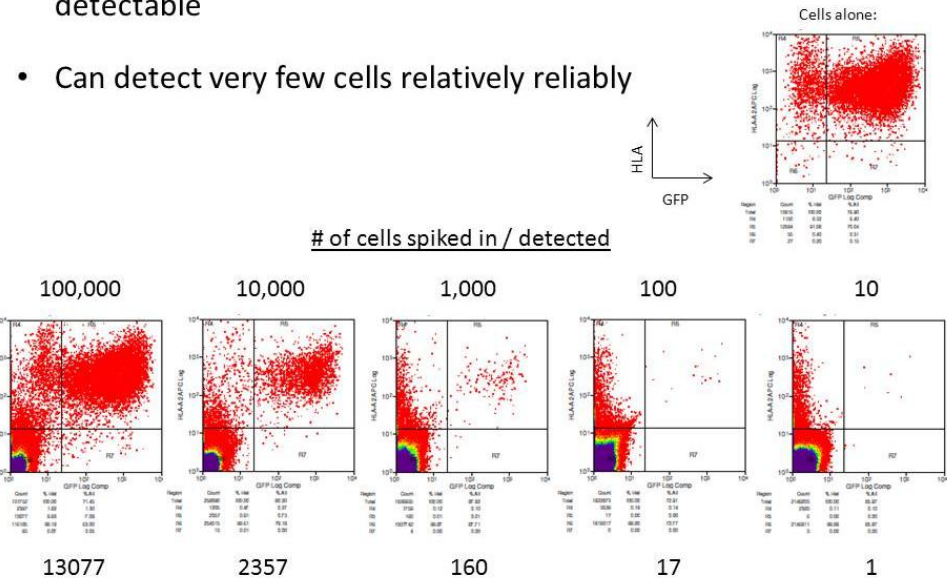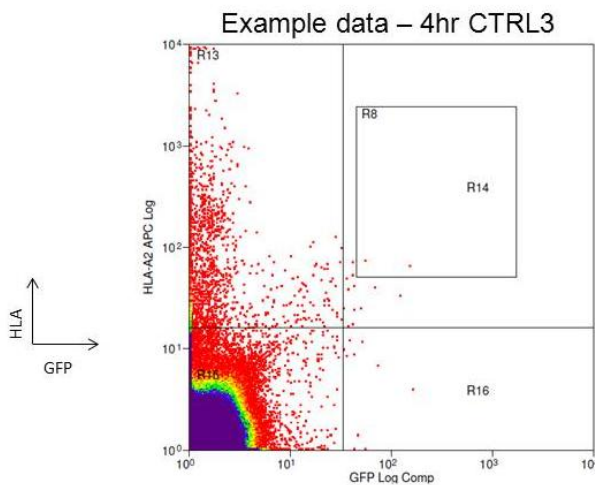- Can detect very few cells relatively reliably

Figure 8. Proof of principal testing of flow cytometric analysis method to identify human breast cancer cells in mouse blood. To confirm that we would be able to detect small numbers of our experimentally-injected cells in the circulation, the indicated numbers of BT549 cells expressing GFP were added to 1ml of mouse whole blood collected by cardiac puncture. Samples were then subjected to red blood cell lysis and staining, and analyzed by flow cytometry for the presence of human HLA-A2+/GFP+ cells.

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We then used the BT549 TripZ-200c cells cultured for 48 hours in normal culture media, or in the presence of doxycycline to induce expression of miR-200c. Cells were trypsinized, washed, counted, and resuspended in PBS at 5x10^6 cells per mL. 100ul of PBS (500,000 cells) was injected into the tail vein of each nude mouse (4 or 5 mice per group). Whole blood was collected via cardiac puncture 4 and 24hrs later. Blood was incubated at room temperature with red blood cell lysis buffer for 10 minutes, spun down, and pellets were resuspended in 100ul of FACS staining buffer. Samples were stained for 30 minutes on ice with 1ng/mL DAPI and antibodies against Mouse CD45-PE and Human HLA-II-APC. Samples were then washed with PBS and resuspended in FACS buffer for analysis (Figure 9).

Figure 9. In vivo anoikis resistance experiment. *24hrs before injection, BT549 cells were treated either with vehicle (CTRL) or 1ug/ml Doxycycline (200c) to induced expression of miR-200c. 500,000 cells in 100ul PBS were injected into the tail vein of nude mice, and whole blood was collected by cardiac puncture at the indicated time thereafter. The DAPI staining of cells which stained positively for human-specific HLA-A2 was assessed in each individual sample. A ratio of HLA+/DAPI- (live) and HLA+/DAPI+ (dead or dying) cells was then calculated (A) and the values for each group were averaged (B). p<.05 for 4hr timepoint*
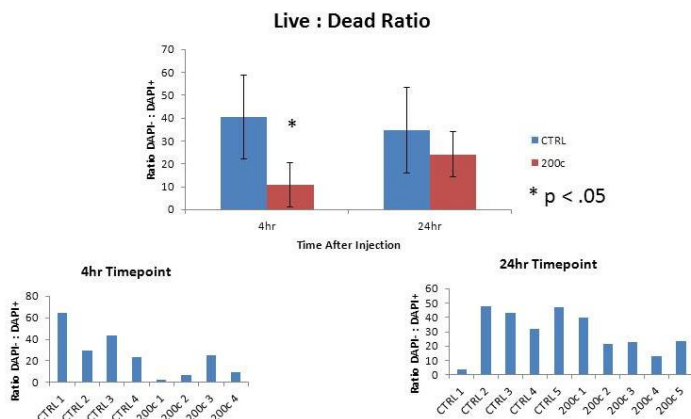
- In actuality, almost no HLA+/GFP+ cells detected
  - Possibly due to loss of GFP signal by cells during early stages of apoptosis leading to leaky cells.

In order to control for the variable amount of blood obtained from each animal, we chose to look at the ratio of live to dead/dying human cells in our samples. We found very few GFP+/HLA+ cells (see below for possible explanation), therefore we focused our analysis on cells that stained positively for the human HLA cell surface antigen. The ratio of HLA+/DAPI- (live) to HLA+/DAPI+ (dead or dying) cells was calculated for each sample, and the experimental groups were averaged. At 4 hours post-injection, animals receiving control BT549 cells had a higher ratio of live:dead cells than did the miR-200c-expressing BT549 cells. At 24 hours post-injection, the results showed a similar trend, with animals receiving control cells having a higher ratio of live:dead cells, however the difference did not reach significance at this timepoint. Taken together, our results suggest that, as we hypothesized, restoration of miR-200c expression in triple-negative breast cancer cells increases their sensitivity to anoikis in the circulation (Figure 10).

BT549 in vivo anoikis experiment

- Ratio of HLA+/DAPI- (live) and HLA+/DAPI+ (dead/dying) cells was obtained in each sample
- CTRL cells have higher amount of live to dead cells at both time points compared to 200c-expressing cells, suggesting that miR-200d is enhancing anoikis (detachment induced death).

Figure 10. Restoration of miR-200c does result in enhanced cell death (anoikis) in vivo (resulting in significantly fewer) cells surviving after 4 hours in the bloodstream. *BT549 cells were treated either with vehicle (CTRL) or*

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1ug/ml Doxycycline (200c) to induced expression of miR-200c for 24hrs before injection. 500,000 cells in 100ul PBS were injected into the tail vein of nude mice, and whole blood was collected by cardiac puncture at the indicated time thereafter. The DAPI staining of cells which stained positively for human-specific HLA-A2 was assessed in each individual sample. A ratio of HLA+/DAPI- (live) and HLA+/DAPI+ (dead or dying) cells was then calculated (A) and the values for each group were averaged (B). Student's t-test was significant for the 4 hr time point p<.05. In the 24 hr timepoint there was one outlier of the 4 mice that was more than 2 standard deviations from the rest of the mice in that group, so that one was left out of the analysis. Results were still not significant.

We additionally set up another experiment where 4 mice per group received 150,000 cells per mouse (4 mice with miR-200c induced cells injected into the tail vein and 5uninduced) and these were let live for 2 weeks. At two weeks we harvested the lungs to determine which group (the induced BT549s to which miR-200c is restored) or the uninduced, which lack miR-200c) will have fewer tumor colonies in the lungs. Mice were euthanized by approved methods and lungs were perfused with 10% formalin at the time of collection and fixed overnight in 10% formalin, then washed in 70% ethanol and paraffin embedded. We used Millipore MAB3580 for our staining (mouse monoclonal). This experiment will answer the question as to whether miR-200c causes anoikis sensitivity so that fewer cells can arrive at distant metastatic sites by surviving the trip through the bloodstream. We ran out of time to finish the analysis on these samples before this progress report was due. The initial staining with GFP on the formalin fixed paraffin embedded lungs used a mouse monoclonal antibody which is showing a lot of background, so our plan is to find tumor cells by H&E staining (our collaborating pathologist, Dr. Paul Jedlicka will examine), then stain with a non-mouse generated anti-GFP antibody. We can then perform a quantitative analysis looking at multiple sections from each mouse to determine which group ultimately has the most tumor colonies in the lungs.

Future modifications to the protocol. As far as we can tell, this is the first study to isolate and test the anoikis resitance step in the metastatic cascade *in vivo*. Therefore we were on our own in designing this protocol. In order to confirm and expand upon our results, we plan to repeat this experiment with modifications to our assay design. One problem that was encountered with our assay is that dying cells tend to rapidly lose GFP because they become leaky. Thus our proposed method of identifying the injected experimental cells as GFP+/HLA+ then assessing apoptosis is not ideal, as we may be missing cells undergoing apoptosis that display decreased GFP fluorescence. Instead, in future experiments we will label cells with a stable fluorescent tracking molecule such as CellTracker Green, which is retained inside cells after incubation in culture and is strongly fluorescent for up to 72hrs, allowing for more reliable detection of experimental cells in the circulation. Additionally, we found that the DAPI fluorescence was relatively dim, and may only be staining cells in the late stages of cell death. In future experiments, we will instead assess apoptosis by staining with Annexin V, which stains cells early in the apoptotic process and should give a stronger fluorescent signal. Finally, due to the rapid loss of cells from the circulation (by being physically trapped in capillaries and the liver), it may be informative to look at the viability of cells in organs such as the liver. In future experiments (hopefully funded by an idea expansion grant), we will collect both lungs and liver from animals at all timepoints and analyze them for the presence and viability of tumor cells by immunofluorescence.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

1) Determined that restoration of miR-200c dramatically reduces migration and invasion of breast cancer cells in vitro by targeting of FN1 and MSN.
2) Identified anoikis resistance as an additional marker of the EMT phenotype and identified the target that mediates the ability of miR-200c to reverse anoikis resistance in TNBC as NTRK2.
3) Have identified two breast cancer cell lines that may be resistant to taxanes via this mechanism.

4) Designed primers to determine if miR-200c is silenced by methylation in some cell lines such that even if ZEB1 is repressed, miR-200c is not re-expressed. Can sequence the products of these primers to see if miR-200c binding sites are mutated.

5) Determined by FISH analysis that a large chromosomal deletion is not the means by which miR-200c is lost in TNBC.

6) Obtained IRB approval to perform miR-200c in situ hybridization on clinical samples. But probe does not work.

7) Made inducible miR-200c in the TNBC line BT549 cells. Picked clone that gives good expression and activity. Have not had success picking one from MDA-MB-231s that make enough miR-200c when its induced with doxycicline, but is not leaky in the uninduced state.

8) Demonstrated that TrkB and its ligand are increased in breast cancer cells in forced suspension due to an increase in NFkB activity on their promoters and the transcripts are allowed to be translated when miR-200c is not present, but directly repressed when it is. This is the first demonstration of an inappropriate autocrine loop in TNBCs that leads to anoikis resistance. This process can be reversed by miR-200c and there are also TrkB inhibitors available now, but we have not yet tried them.

9) Made new protocol to isolate human tumor cells quickly from mouse blood to test our theory that miR-200c .

10) Conducted in vivo experiment to determine if miR-200c affects anoikis resistance in vivo in mice.

REPORTABLE OUTCOMES:

Manuscripts:

1. Cochrane DR, Spoelstra NS, Nordeen SK and **JK Richer**. MicroRNA-200c Mitigates Invasiveness and Restores Sensitivity to Microtubule-Targeting Chemotherapeutic Agents. MOL CANCER THER. 2009 May;8(5):1055-66. PMID:19435871

2. Cochrane DR, Cittelly DM, Howe EH., Spoelstra NS, McKinsey EL, LaPara K, Elias A, Yee D, and **JK Richer**. MicroRNAs Link Estrogen Receptor alpha Status and Dicer Levels in Breast Cancer. HORMONES AND CANCER. 2010. 1(6): 306-319. PMID:21761362

3. Howe, E. N. Dawn R. Cochrane and **J.K. Richer**. Targets of miR-200c mediate suppression of cell motility and anoikis. BREAST CANCER RES. 2011 Apr 18;13(2):R45. PMID: 21501518.

*Highlighted by DC Radisky BREAST CANCER RES Jun 10;13(3):110 PMID: 21501518

4. Howe EN, Cochrane DR and **Richer JK**. The miR-200 and miR-221/222 miRNA Families: Opposing Effects on Epithelial Identity. J of MAMMARY GLAND BIOLOGY AND NEOPLASIA. 2012 Feb 17 epub ahead of print PMID: 22330642.

5. Howe EN, Cochrane DR, Cittelly DM and **Richer JK**. miR-200c targets a TrkB/NTF3 autocrine signaling loop to enhance anoikis sensitivity in triple negative breast cancer. PLOS One 2012;7(11). PMID: 23185507.

6. D'Amato, NC**,** EN Howe, **JK Richer**. MicroRNA regulation of epithelial plasticity in cancer. Invited review, In Press, Cancer Letters (2012)

Review with no new data, so did not cite grant:

Wright J, **Richer JK** and Goodall GJ. MicroRNAs and EMT in mammary cells and breast cancer. J MAMMARY GLAND BIOLOGY AND NEOPLASIA. 15(2):213-23. 2010. PMID:20499142

Abstracts (Poster Presentations)

Erin N. Howe and Jennifer K. Richer. miR-200c Targets a TrkB/NTF3 Autocrine Signaling Loop to Suppress Anoikis Resistance. AACR Special Conference on Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications. October 2011.
Erin N. Howe, Dawn R. Cochrane and Jennifer K. Richer. miR-200c Directly Targets Multiple Non-epithelial GenesInvolved in Motility and Anoikis Resistance. Rocky Mountain Reproductive Sciences Symposium. April 2010.

Erin N. Howe, Dawn R. Cochrane and Jennifer K. Richer. Targets of miR-200c Mediate Suppression of Cell Motility and Anoikis Resistance. Program in Cancer Biology annual retreat. January 2011.

Dawn R. Cochrane, Erin N. Howe, Erin McKinsey, Britta M Jacobsen, Steven M. Anderson, and Jennifer K. Richer. MiRNAs that Define Breast Cancer Phenotypes. Endocrine Society Annual meeting, June 2010.

Dawn R. Cochrane, Erin H. Howe, Diana M. Cittelly, Nicole S. Spoelstra, Erin L McKinsey, Anthony Elias and Jennifer K. Richer. EMT associated miRNAs control Dicer expression. Keystone Epithelial Plasticity and Epithelial to Mesenchymal Transition, January 2011.

Presentations

Richer, JK, EN Howe, DR Cochrane**,** and D Cittelly. Invited lecture "MicroRNAs that Regulate EMT in Breast and Gynecological Carcinomas" for Educational Section on EMT and Stem Cells in Cancer Progression. **AACR 102nd Annual Meeting**. Orlando, FL. April, 2011

Richer, JK. Invited lecture "MicroRNAs that regulate EMT and tumor progression in gynecological cancers" for the **MD Anderson Annual Uterine Cancer Biology Symposium** for the MD Anderson Gynecologic Cancer SPORE, May 19-20th, 2011.

Richer, JK. Invited lecture **"**miRNAs as guardians of the epithelial phenotype" **Gordon Conference on Mammary Gland Biology**. June, Newport, RI, 2011

Richer, JK. Invited Lecture "MicroRNAs Regulate Endometrial and Ovarian Cancer Sensitivity to Anoikis and Chemotherapy" for Symposium on Reproductive Cancers. **Society for the Study of Reproduction Annual Meeting**. July Portland, OR, 2011

Richer, JK. Invited Lecture "MicroRNAs and Hormone Receptor Action in Breast Cancer" for the **Annual Endocrine Society Meeting** Symposium "Putting the Brakes on Breast Cancer." Houston, TX, June, 2012

Howe, EN, DR Cochrane and JK Richer**.** Insurance against EMT: MiR-200c directly targets multiple nonepithelial genes. Graduate Student **Invited Short Talk.** AACR EMT and Cancer Progression and Treatment. February, 2010

Erin N. Howe, Dawn R. Cochrane and Jennifer K. Richer. Identification of Direct Targets of miR-200c and Their Contribution to Progression of Breast and Endometrial Cancer. Department of Pathology, Grand Rounds. February 2010.

Erin N. Howe and Jennifer K. Richer. Affect of Restoration of miR-200c on Breast Cancer Cell Polarity in 3-D Culture. University of Colorado, Anschutz Medical Campus Program Project Grant on Normal Mammary Gland Development retreat. January 2011.

> **Erin N. Howe**, Dawn R. Cochrane and Jennifer K. Richer. Targets of miR-200c Mediate Suppression of Migration and Anoikis Resistance. Department of Pathology, Research in Progress. November 2010.

> **Dawn R. Cochrane**, Erin N. Howe, Diana M. Cittelly, Nicole S. Spoelstra, Erin L McKinsey, Anthony Elias and Jennifer K. Richer. MiRNAs that shape breast cancer phenotypes. University of Colorado Cancer Biology Graduate Program Retreat, January 2011. Oral Presentation.

> **Richer, JK,** EN Howe, and DR Cochrane. **Invited Short Talk.** "MicroRNAs Differentially Expressed in Luminal versus Triple Negative Breast Cancer Control Estrogen Receptor alpha and Growth Factor Receptor Expression and Aspects of Tumor Metabolism." Keystone Symposia: Nuclear Receptors: Signaling, Gene Regulation and Cancer. March, 2010.

Honors and awards:

- Gary J. Miller award for outstanding research by a graduate student in the Department of Pathology.

Degrees conferred:

Erin Howe, Doctorate in Cancer Biology Graduate Program, University of Colorado July, 2012

Patents

- 2009   U.S. Provisional Application for United Stats Letters Patent *UTEC*:021USP1 "Micro RNAs Dysregulated in Triple-Negative Breast Cancer Inventors: Jennifer Richer, Dawn Cochrane, Steve Anderson

Development of cell lines, tissue or serum repositories;

Funding applied for based on work supported by this award:

DOD BCRP- Idea Expansion
Supporting Agency: Department of Defense Breast Cancer Research Program
Role: PI   **Level of effort:** 25%

Title: Reversing Anoikis Resistance in Triple Negative Breast Cancer

Hypothesis: We postulate that loss of miR-200c and overexpression of miR-222 are largely responsible for the dedifferentiated phenotype and aggressive behavior of claudin low TNBC and metaplastic breast cancers and that manipulation of these miRNA or key targets could render such tumors less aggressive **and more**

treatable. We have designed aims that will take our original innovative findings to the next level toward impacting breast cancer patients.**Goal:** To test the hypothesis that loss of miR-200c and overexpression of miR-222 are responsible for the dedifferentiated phenotype and aggressive behavior of claudin low TNBC and metaplastic breast cancers and that manipulation of these miRNA or key targets could render such tumors less aggressive and more treatable**.**

Aim1: Determine if miR-200c and inhibition of miR-222 in TNBC can enhance differentiation in 3D culture.''

Aim 2: Identify the mechanisms by which TNBC cells resist anoikis.

Aim 3: Utilize recently derived basal like TNBC cell lines and patient-derived tumor explants to determine if manipulation of miR-200c and 222 will render TNBC less aggressive *in vivo* following manipulation of miRNAs that control differentiation, anoikis- and chemo-sensitivity.

Erin Howe NRSA pre-doctoral fellowship, miR-200c Targets a TrkB/NTF3 Autocrine Signaling Loop to Suppress Anoikis Resistance.F31CA165668-01.

Employment or research opportunities applied for and/or received based on experience/training supported by this award

Erin Howe has started a post-doctoral fellowship position with Dr. Carrie Rinker-Schaeffer at the University of Chicago, and began her work there following her thesis defense.

CONCLUSION:

Significance of our findings and impact of the proposed research on breast cancer: The beauty of miR-200c is that it suppresses multiple targets involved in tumor progression. It reverses resistance to taxanes (9), inhibits migration and invasion by targeting *ZEB1/2, FN,* and *MSN (18)*, and enhances anoikis sensitivity, a potent barrier to the multistep process of metastases by targeting TrkB and NTF3 (9,10). We propose miR-200c and miR-222 inhibition as "differentiation therapy" that would have presumably low toxicity since it is normally expressed in epithelium and inhibits genes that should not be expressed in normal epithelial cells. Although in this proposal we use lentiviral delivery of miRNA as proof of principal, we hope to obtain future funding to test non-viral delivery methods via a partnership with Dr. Tom Anchordoquy. Expansion of the original innovative research will determine the extent to which these miRNAs can alter the phenotype and behavior of TNBC and cause differentiation or death of tumor cells at critical steps in the metastatic cascade. Even if systemic delivery of miRNAs for breast cancer treatment proves impossible, our experiments will unveil new important druggable pathways used by TNBC to resist anoikis. For instance TrkB inhibitors or inhibitors of the other new pathways that we find TNBC exploiting to resist anoikis and metastasize.

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APPENDICES:

Manuscripts 1-6 are contained in this appendix.

miR-200c Targets a NF-κB Up-Regulated TrkB/NTF3 Autocrine Signaling Loop to Enhance Anoikis Sensitivity in Triple Negative Breast Cancer

Erin N. Howe[1,2¤], Dawn R. Cochrane[2], Diana M. Cittelly[2], Jennifer K. Richer[1,2]*

1 Program in Cancer Biology, University of Colorado, Anschutz Medical Campus, Aurora, Colorado, United States of America, **2** Department of Pathology, University of Colorado, Anschutz Medical Campus, Aurora, Colorado, United States of America

Abstract

Anoikis is apoptosis initiated upon cell detachment from the native extracellular matrix. Since survival upon detachment from basement membrane is required for metastasis, the ability to resist anoikis contributes to the metastatic potential of breast tumors. miR-200c, a potent repressor of epithelial to mesenchymal transition, is expressed in luminal breast cancers, but is lost in more aggressive basal-like, or triple negative breast cancers (TNBC). We previously demonstrated that miR-200c restores anoikis sensitivity to TNBC cells by directly targeting the neurotrophic receptor tyrosine kinase, TrkB. In this study, we identify a TrkB ligand, neurotrophin 3 (NTF3), as capable of activating TrkB to induce anoikis resistance, and show that NTF3 is also a direct target of miR-200c. We present the first evidence that anoikis resistant TNBC cells up-regulate both TrkB and NTF3 when suspended, and show that this up-regulation is necessary for survival in suspension. We further demonstrate that NF-κB activity increases 6 fold in suspended TNBC cells, and identify RelA and NF-κB1 as the transcription factors responsible for suspension-induced up-regulation of TrkB and NTF3. Consequently, inhibition of NF-κB activity represses anoikis resistance. Taken together, our findings define a critical mechanism for transcriptional and post-transcriptional control of suspension-induced up-regulation of TrkB and NTF3 in anoikis resistant breast cancer cells.

Introduction

Breast cancer is the most commonly diagnosed malignancy among American women, and an estimated 226,870 women will be diagnosed in 2012 [1]. Although the mortality rate for breast cancer has improved, it remains the second most deadly cancer for American women, with mortality largely attributed to metastatic disease [2]. Metastasis is a complicated process, during which cells must undergo dramatic phenotypic changes to migrate away from the primary tumor, survive in the vasculature or lymphatics, and finally colonize metastatic sites. Oncogenic epithelial to mesenchymal transition (EMT) is thought to play an important role in the ability of cells to acquire traits necessary to metastasize [3]. The miR-200 family of miRNAs has emerged as a potent regulator of EMT. miRNAs are small (18–25 nucleotide) non-coding RNAs that regulate gene expression post-transcriptionally by binding to the 3′ untranslated region (UTR) of the target mRNA [4], and inhibiting translation or targeting the mRNA for degradation [5]. The miR-200 family is comprised of two polycistronic clusters – miR-200c and miR-141 on chromosome 12 and miR-200b, miR-200a and miR-429 on chromosome 1. The miR-200 family is highly expressed in breast epithelial cells and luminal-like carcinomas, and lost in the more aggressive basal-like, or triple negative carcinomas [6,7]. These miRNAs serve to maintain an epithelial phenotype and protect against EMT through repression of multiple targets, including the EMT-inducing transcription factors – ZEB1 and ZEB2 [8–10], genes involved in migration – FN1, MSN [11], and WAVE3 [12], and epigenetic regulators – SIRT1 [13], and Suz12 [14].

In addition to enhanced migratory and invasive capacity necessary for metastasis, cells must also resist anoikis while in transit to metastatic sites. Anoikis is apoptosis initiated by loss of attachment to the native extracellular matrix (ECM) [15,16], and has been suggested as a physiological barrier to metastasis [16–19]. Anoikis resistance correlates tightly with an EMT phenotype [20–24]. We previously demonstrated that restoration of miR-200c to aggressive triple negative breast (TNBC) and Type 2 endometrial cancer cell lines significantly enhances anoikis sensitivity [11]. Furthermore, we identified the neurotrophic tyrosine kinase, type 2 (NTRK2 or TrkB) as a direct target of miR-200c, and demonstrated that addition of untargetable TrkB reversed the ability of miR-200c to sensitize TNBC cells to anoikis [11].

TrkB plays a crucial role in the formation and function of the nervous system [25,26], including the promotion of neuronal survival [27]. TrkB was identified as a potent anoikis suppressor in a genome-wide screen for genes capable of conferring anoikis

resistance to rat intestinal epithelial cells [28]. Indeed, TrkB and BDNF induce anoikis resistance in a variety of carcinoma models including breast [29], ovarian [30,31], and head and neck [32]. However, neurotrophin 3 (NTF3) also activates TrkB [33], and is a predicted target of miR-200c. In this study, we present the novel finding that TNBC cells in suspension up-regulate both TrkB and NTF3 to enable anoikis resistance. We find that NF-κB drives transcription of both TrkB and NTF3, and that miR-200c potently suppresses anoikis resistance by directly targeting both components of this aberrant autocrine signaling loop.

Results

TNBC Cells are More Anoikis Resistant than Luminal A Cells and miR-200c Sensitizes TNBC Cells to Anoikis

We utilize four breast cancer cell lines to dissect miR-200c-mediated control of anoikis resistance. MDA-231 and BT549 cell lines are representative TNBC cell lines. They are motile and invasive in culture, and able to metastasize from an orthotopic site. Genetically they represent the basal B subtype [34]. They do not express estrogen receptor alpha (ERα), progesterone receptors (PR) or Her2/Neu, nor do they express the epithelial adherens junction protein E-cadherin or the miR-200 family, instead expressing mesenchymal markers such as N-cadherin and vimentin. MCF7 and T47D cells represent the luminal A subtype, expressing epithelial markers such as ERα, E-cadherin, and the miR-200 family. They are weakly invasive in culture and tumorigenic when provided with estrogen, but do not typically metastasize from the orthotopic site.

We show that the less aggressive MCF7 and T47D cells are strongly sensitive to anoikis following 24 hrs in suspension, as demonstrated by strong staining with propidium iodide (PI) (Fig. 1A, Left). The MDA-231 and BT549 cells, however, show little apoptosis following culture in suspension (Fig. 1A, Left). When the proportion of PI to DAPI staining is quantitated, the luminal lines exhibit twice the amount of apoptosis observed in TNBC lines (Fig. 1A, Right). We next sought to determine if restoring miR-200c to the TNBC cells would sensitize them to anoikis to the extent observed in luminal lines. The levels of miR-200c attained following transfection of the mimic into the TNBC cells match the levels endogenously expressed in luminal lines (Fig. 1B). We find that restoration of miR-200c induces cell death in suspended TNBC cells (Fig. 1C). Taken together, this data indicates that miR-200c represses genes that TNBC cells require to resist anoikis, and expression of miR-200c in these cells sensitizes them to anoikis.

TrkB Requires Ligand to Induce Anoikis Resistance

TrkB is a cell surface receptor tyrosine kinase, and as such is activated by ligand binding. BDNF is the preferred ligand of TrkB in a neuronal setting [35]; however, it is not the only neurotrophic factor capable of activating TrkB. NTF3 also binds and activates TrkB [33], and NTF3 is a predicted target of miR-200c. To determine if BDNF or NTF3 activate TrkB signaling in a breast cancer model, we stably transfected empty vector (EV) or TrkB into MCF7 and T47D cells (Fig. 2A). The cells were then plated in suspension in medium containing increasing concentrations of BDNF and NTF3 (Fig. 2B, C). Addition of BDNF has no effect on EV expressing cells, but increases the anoikis resistance of TrkB expressing cells as expected, as indicated by decreased cell death. Likewise, treatment with NTF3 induces anoikis resistance of TrkB expressing cells to the same extent as BDNF. Of note is the fact that neither ligand affected survival of EV or TrkB expressing adherent cells (Fig. S1), indicating that activation of TrkB by

BDNF or NTF3 affects anoikis specifically. Thus, in breast cancer cells, NTF3 is capable of activating TrkB to induce anoikis resistance to the same extent as BDNF, supporting our hypothesized role of NTF3 in TrkB-mediated anoikis resistance.

NTF3 is a Direct Target of miR-200c

The 3′ UTR of NTF3 contains two putative miR-200c binding sites (Fig. 3A). We cloned the region containing these sites downstream of luciferase in a reporter plasmid. We observe a 35% decrease in luciferase activity following introduction of miR-200c, with no decrease in mock transfected or negative controls (Fig. 3B). When mutations are made in the putative miR-200c binding sites, luciferase activity returns to control levels, indicating that binding to either of these specific sites is required for down-regulation. When an antagomiR is used to inhibit miR-200c binding, luciferase activity is again restored. This indicates that miR-200c specifically is responsible for binding to the 3′ UTR. Together this data shows that miR-200c binds to two specific sites in the NTF3 3′ UTR to down-regulate reporter activity. Importantly, restoration of miR-200c to MDA-231 and BT549 cell lines leads to a significant decrease in the amount of secreted NTF3 (Fig. 3C). Thus, restoration of miR-200c to two TNBC cell lines significantly represses expression of NTF3 through direct targeting.

miR-200c Suppresses Anoikis Resistance through Targeting of the TrkB/NTF3 Signaling Axis

Having shown that the combination of TrkB and NTF3 is sufficient to induce anoikis resistance in luminal breast cancer cells, we sought to determine if TrkB and NTF3 are necessary for TNBC cells to resist anoikis. To answer this question, we utilized shRNA constructs against TrkB and NTF3 to determine if knockdown of either component of the signaling loop would sensitize cells to anoikis. BT549 cells were stably selected for expression of shRNAs targeting TrkB or NTF3. shTrkB 2242 was most effective at knocking down TrkB expression with a 56% reduction (Fig. 4A), while shNTF3 58854 knocked down NTF3 most efficiently with a 60% reduction (Fig. 4B). To determine the effect of the shRNAs on anoikis resistance, cells were plated in suspension and harvested at 24 hrs. We show that expression of either shTrkB construct induces cell death, with shTrkB 2242 inducing a strong 80% increase in cell death, indicating a decrease in anoikis resistance (Fig. 4C). Similarly, both shNTF3 constructs decrease anoikis resistance, with shNTF3 58854 inducing the strongest decrease (Fig. 4C). Similar results were obtained in the MDA-231 cell line (Fig. S2). Overall, knockdown of either TrkB or NTF3 significantly decreased the ability of these cells to survive in suspension; thus, TrkB and NTF3 are necessary for BT549 and MDA-231 cells to resist anoikis.

To determine if suppression of TrkB and NTF3 is the mechanism by which miR-200c suppresses anoikis resistance, BT549 cells expressing shTrkB and shNTF3 constructs were transfected with miR-200c mimic and plated in suspension. We find that cells expressing the less effective shTrkB 195114 construct exhibit further repression of TrkB expression when transfected with miR-200c (Fig. 4D). Cells expressing shTrkB 2242 do not show further repression of TrkB when expressing miR-200c (Fig. 4D), indicating that miR-200c suppresses TrkB expression as effectively as shTrkB 2242. Similarly, cells expressing shNTF3 58853 secrete less NTF3 into the medium when transfected with miR-200c, while cells expressing shNTF3 58854 do not (Fig. 4E), again indicating that miR-200c suppresses NTF3 expression as effectively as shNTF3 58854. We next investigated the presence of additive effects between the shRNA constructs and miR-200c to determine if repression of TrkB and NTF3 signaling is the
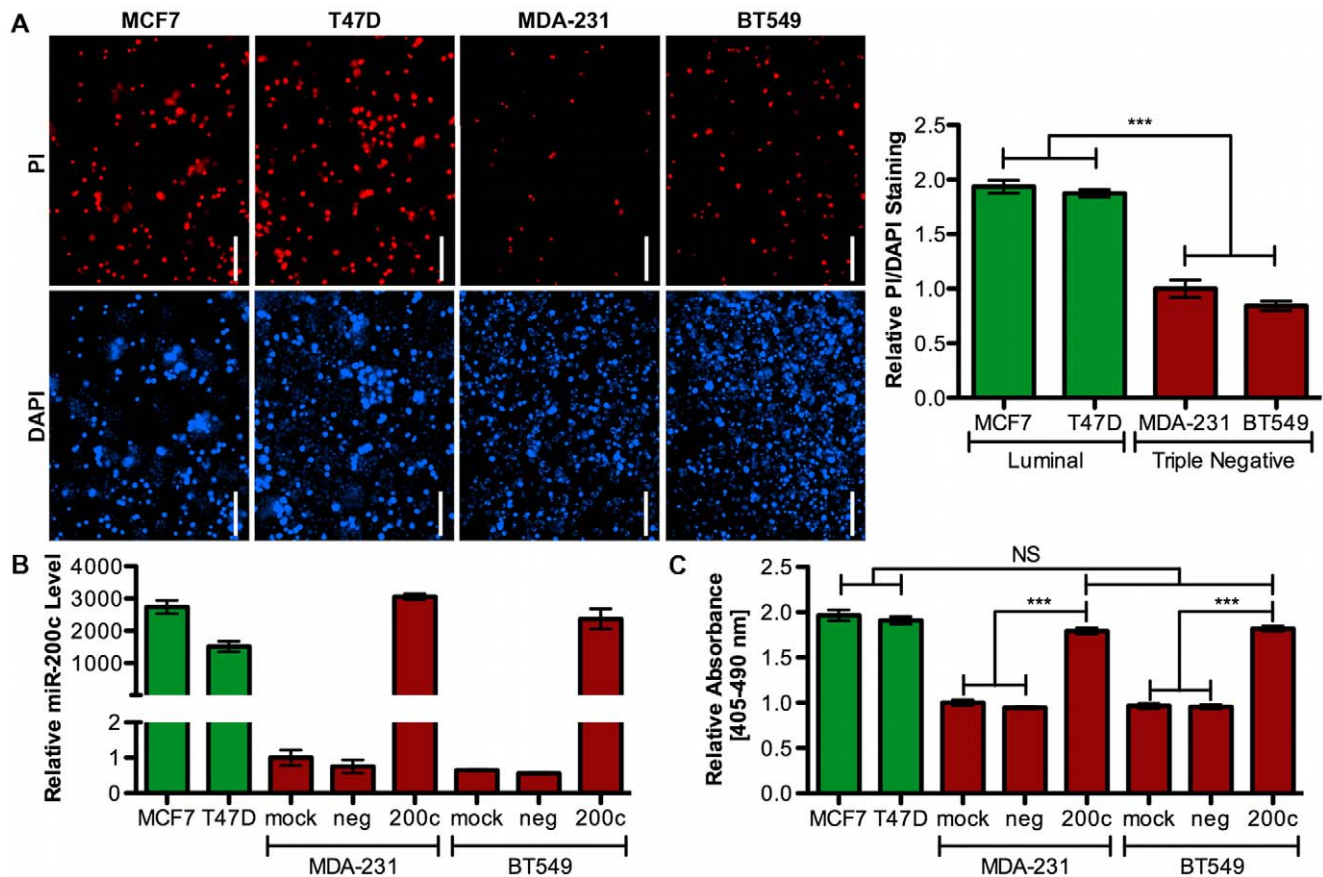
Figure 1. Triple negative breast cancer cells are more anoikis resistant than luminal cells and miR-200c sensitizes aggressive cells to anoikis. A. Cells were plated attached or suspended for 24 hrs prior to staining with DAPI and propidium iodide (PI). Representative images of suspended cells are shown, scale bar 50 µm. Quantitation of data in A, presented as a ratio of PI to DAPI, with each cell line normalized to the attached condition. Shown relative to MDA-231 cell line. *Columns*, mean of three biological replicates, *bars*, SEM. **B.** Cells treated with transfection reagent only (mock), scrambled negative control (neg) or miR-200c mimic (200c) and 48 hrs later harvested for qRT-PCR analysis of miR-200c levels. Data normalized to U6 levels and presented relative to MDA-231 mock transfection condition. *Columns,* mean of five biological replicates, *bars,* SEM. **C.** Cells as in B and 24 hrs later plated in suspension. After 24 hrs in suspension, a cell death ELISA was performed. Data normalized to attached condition and shown relative to MDA-231 mock transfection. *Columns*, mean of three biological replicates, *bars*, SEM.
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mechanism by which miR-200c suppresses anoikis resistance. We find that cells expressing shTrkB 2242 construct do not exhibit increased anoikis sensitivity following transfection with miR-200c (Fig. 4F), indicating that miR-200c suppresses anoikis resistance by specifically targeting a TrkB-mediated pathway. Supporting this hypothesis, shNTF3 58854 also does not exhibit increased anoikis sensitivity when transfected with miR-200c (Fig. 4G). Similar results were obtained in MDA-231 cells (Fig. S2). Since effective knockdown of either TrkB or NTF3 does not increase anoikis in suspended cells above that of miR-200c alone, we conclude that miR-200c suppresses anoikis resistance by targeting the TrkB/ NTF3 signaling axis.

TrkB and NTF3 are Up-regulated in Suspended Cells

We made the novel observation that both TrkB and NTF3 exhibit increased expression when TNBC cells survive in suspension. We examined TrkB protein in MDA-231 and BT549 cells following culture in suspension, and found dramatic up-regulation of TrkB beginning at 24 and persisting through 72 hrs in suspension in both cell lines (Fig. 5A). Furthermore, we found that the amount of NTF3 secreted into the medium, as determined by NTF3 ELISA, significantly increases in both cells

lines as rapidly as 4 hrs in suspension (Fig. 5B). To determine the effect of miR-200c on suspension-induced up-regulation of TrkB and NTF3, cells were transfected prior to plating in suspension. We found that expression of miR-200c blocked suspension-induced up-regulation of both TrkB (Fig. 5C) and NTF3 (Fig. 5D). Together, this data indicates that anoikis resistant breast cancer cells dramatically up-regulate an autocrine signaling loop following loss of ECM attachment, and restoration of miR-200c blocks the ability to establish this loop. We next sought to determine if TrkB and NTF3 are up-regulated at the transcriptional and/or post-transcriptional level. TrkB and NTF3 mRNA levels increased as early as two hours (Fig. S3), indicating that the up-regulation of these genes is at the transcriptional level.

NF-κB Transcriptional Activity Increases in Suspended TNBC Cells

Examination of the promoter regions of TrkB and NTF3 revealed that both genes contain a number of predicted NF-κB binding sites. NF-κB transcription factors are held inactive in the cytoplasm until the inhibitory IκB complex is proteolytically degraded; thus, NF-κB signaling can be rapidly activated during conditions of cellular stress. Given that TrkB and NTF3 are up-
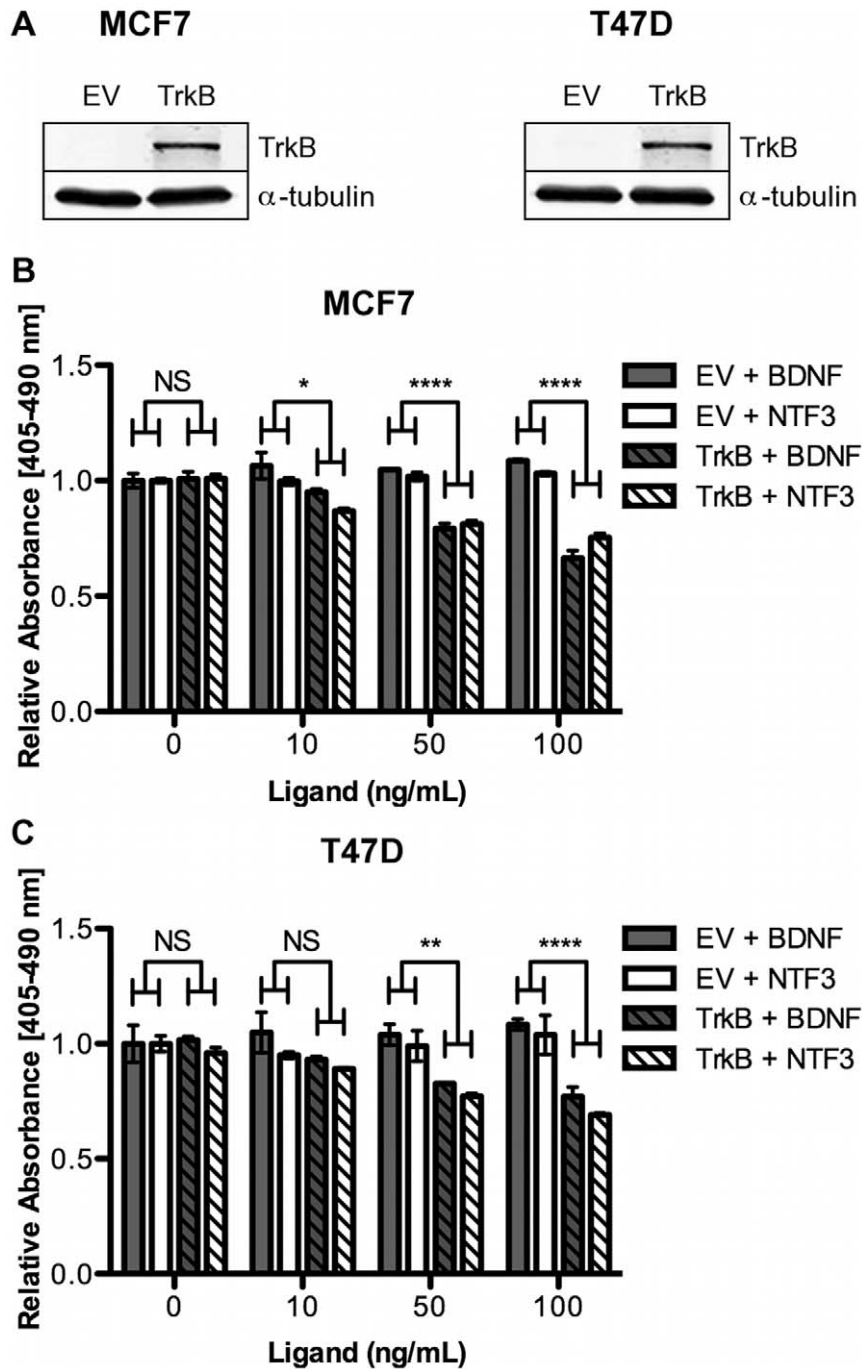
Figure 2. TrkB requires ligand to induce anoikis resistance. A. MCF7 and T47D cells were stably selected for expression of empty vector (EV) or TrkB. Immunoblot showing TrkB expression, α-tubulin used as loading control. MCF7, **B**, and T47D, **C**, cells were plated suspended in increasing concentrations of BDNF or NTF3. Cells were harvested 24 hrs later and apoptosis assayed by cell death ELISA, data normalized to attached condition and shown relative to EV conditions. *Columns*, mean of three biological replicates, *bars*, SEM.
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regulated so quickly, NF-κB signaling was an attractive option to explore. To investigate NF-κB transcriptional activity we used a luciferase reporter containing 3 perfect NF-κB elements upstream of luciferase. We found that anoikis resistant MDA-231 and BT549 cells exhibit higher basal levels of NF-κB transcriptional activity than anoikis sensitive MCF7 and T47D cells, as indicated by increased luciferase activity (Fig. 6A). Importantly, NF-κB transcriptional activity increased dramatically

only in the MDA-231 and BT549 cells during suspension (Fig. 6A). We show that the increased luciferase activity is specific to NF-κB transcriptional activity, since a mutant construct does not exhibit increased activity in the MDA-231 (Fig. 6B) or BT549 cells (Fig. 6C). To confirm these results, we investigated the cellular localization of two NF-κB transcription factors, RelA (p65) and NF-κB1 (p50), in BT549 cells. Both factors are largely cytoplasmic in attached cells, but translocate to the nucleus when cells are
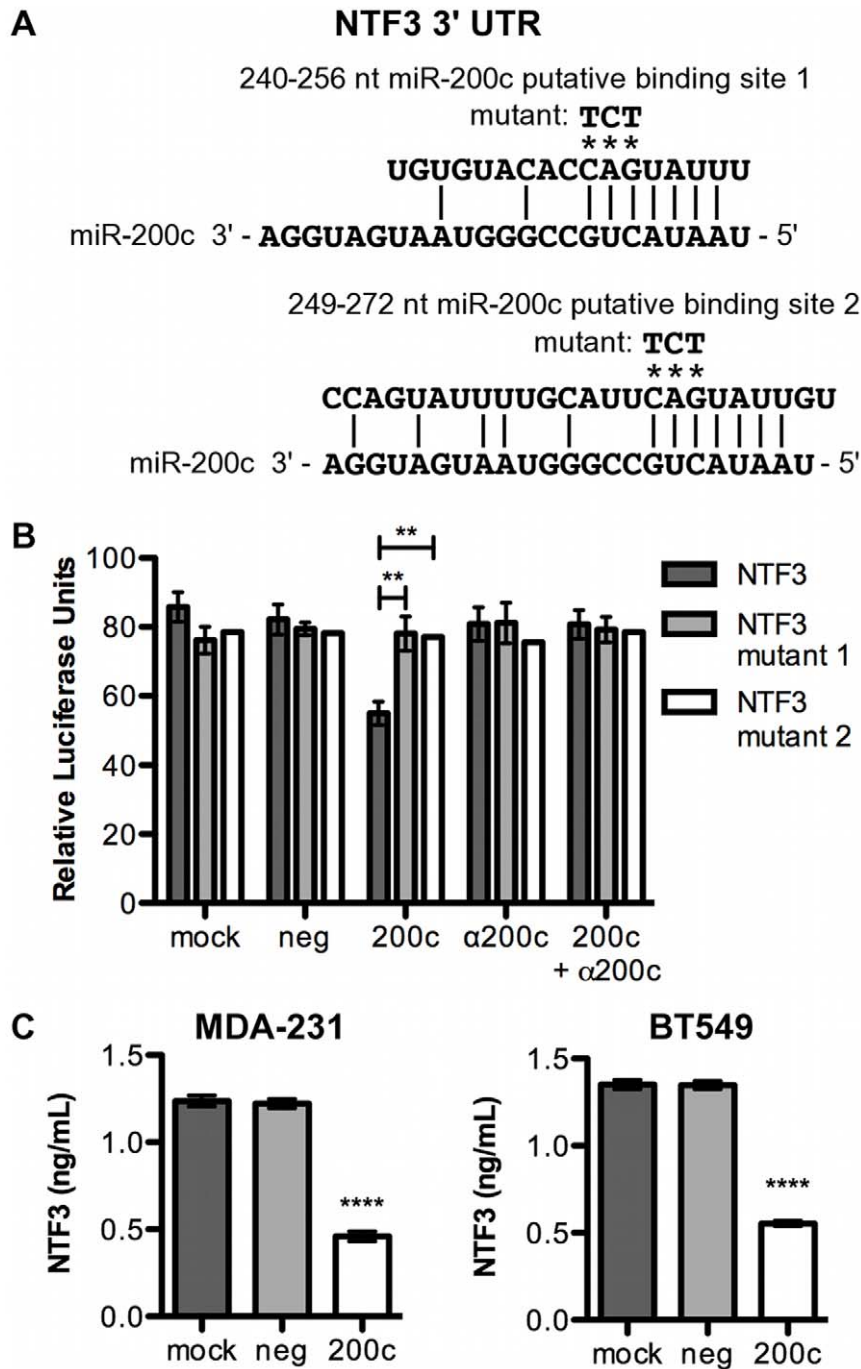
Figure 3. NTF3 is a direct target of miR-200c. A. Regions of the 3′ UTR where miR-200c is predicted to bind. **B.** Hec50 cells transfected with NTF3 luciferase constructs and 24 hrs later treated with transfection reagent only (mock), scrambled negative control (neg), miR-200c mimic (200c), miR-200c antagomiR alone (α200c) or in conjunction with miR-200c (α200c+200c) and luciferase assay performed. *Columns*, mean of five biological replicates, *bars*, SEM. **C**. Cells transfected with miRNA constructs and 48 hrs later medium collected for analysis by NTF3 ELISA. *Columns*, mean of three biological replicates, *bars*, SEM.
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suspended, as indicated by co-localization of DAPI and RelA or NF-κB1 staining (Fig. 6D). Indeed, following 30 minutes in suspension, the percentage of nuclear RelA increases from 6% in attached cells to 70%, while the percentage of nuclear NF-κB1 increases from 21% to 58% (Fig. 6D, Right). Nuclear translocation of NF-κB factors is required for transcriptional activation; thus, this data suggests that these transcription factors are activated during anoikis resistance. Taken together, this data shows that the two NF-κB transcription factors predicted to target the TrkB and NTF3 promoters translocate to the nucleus following loss of ECM attachment, and there is enhanced NF-κB transcriptional activity under these conditions. This suggests that NF-κB transcriptional activity may be involved in suspension induced up-regulation of TrkB and NTF3.
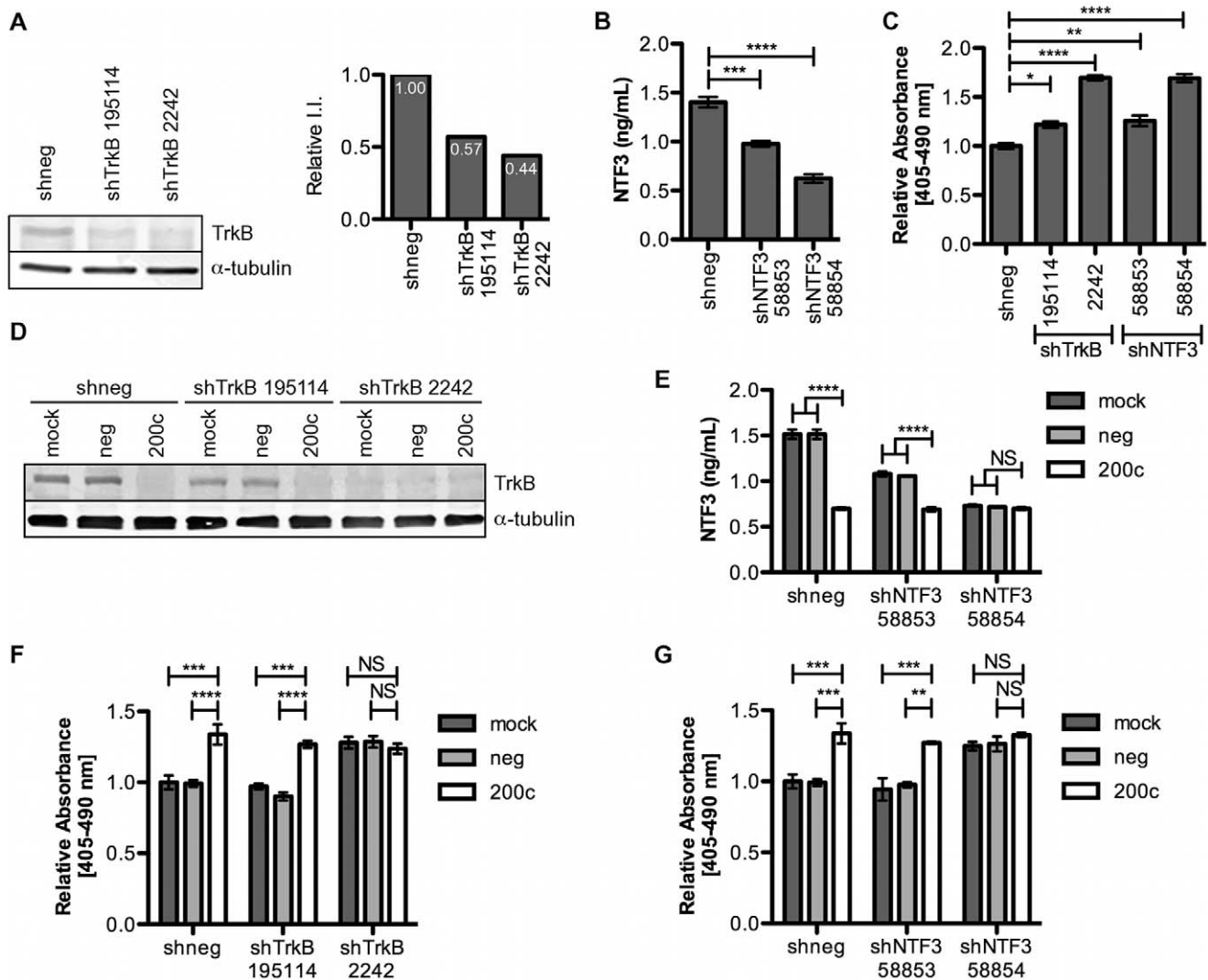
Figure 4. TrkB and NTF3 are required for anoikis resistance. BT549 cells stably selected for expression of shneg, shTrkB or shNTF3 constructs. **A.** Efficacy of TrkB knockdown. *Left*, immunoblot showing knockdown of TrkB, α-tubulin used as loading control, *right*, quantitation of immunoblot. **B.** Efficacy of NTF3 knockdown. NTF3 ELISA performed on medium. *Columns,* mean of three biological replicates, *bars*, SEM. **C.** Cell death ELISA performed on cells suspended for 24 hrs. *Columns,* mean of three biological replicates, *bars*, SEM. **D–G.** Cells treated with transfection reagent only (mock), scrambled negative control (neg) or miR-200c mimic (200c) and 24 hrs later plated in suspension. Cells were harvested 24 hrs later for analysis. **D.** Immunoblot for TrkB, α-tubulin used as loading control. **E.** NTF3 ELISA performed on medium. *Columns,* mean of three biological replicates, *bars*, SEM. shTrkB, **F**, and shNTF3, **G**, cells analyzed by cell death ELISA. *Columns,* mean of three biological replicates, *bars*, SEM.
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NF-κB Transcriptionally Up-regulates TrkB and NTF3 in Suspended Cells

To determine if RelA or NF-κB1 bind directly to regions in the promoters of TrkB and NTF3, and if binding increases when the cells are suspended, we performed chromatin immunoprecipitation (ChIP) on BT549 cells attached, or suspended for 2 hrs. Following IP using antibodies for RelA or NF-κB1, qRT-PCR was performed for 9 regions in the TrkB promoter and 3 in the NTF3 promoter that contain predicted binding sites for RelA or NF-κB1, as indicated by the chart (Fig. 7A, Bottom). Cycle thresholds were first verified to be above those seen in IgG controls (which were unchanged across conditions), and then normalized to the attached condition. The data is presented as a fold enrichment of PCR signal in suspended cells over attached cells, where a signal above 1 indicates that there is enhanced binding of the transcription factor in the region of being amplified. We show

that sites 1 and 5 in the TrkB promoter exhibit increased NF-κB1 binding in suspended cells, while sites 2, 5, 6, and 8 exhibit increased RelA binding (Fig. 7A, Left). Site 1 in the NTF3 promoter exhibits increased NF-κB1 binding, while site 2 exhibits increased RelA binding (Fig. 7A, Right). This data demonstrates that in suspended cells, RelA and NF-κB1 translocate to the nucleus (Fig. 6D), and bind to specific regions in the promoters of TrkB and NTF3 (Fig. 7A).

To examine the effect of NF-κB inhibition on suspension-induced up-regulation of TrkB and NTF3, we utilized a mutant IκBα construct (mIκBα) that cannot be phosphorylated, and thus remains constitutively bound to NF-κB in the cytoplasm, preventing activation of NF-κB transcription. In empty vector expressing cells, IκBα levels decreased from 1.00 to 0.87 when cells are suspended, suggesting proteolysis of IκBα, such as would be expected during activation of NF-κB transcriptional activity. Cells
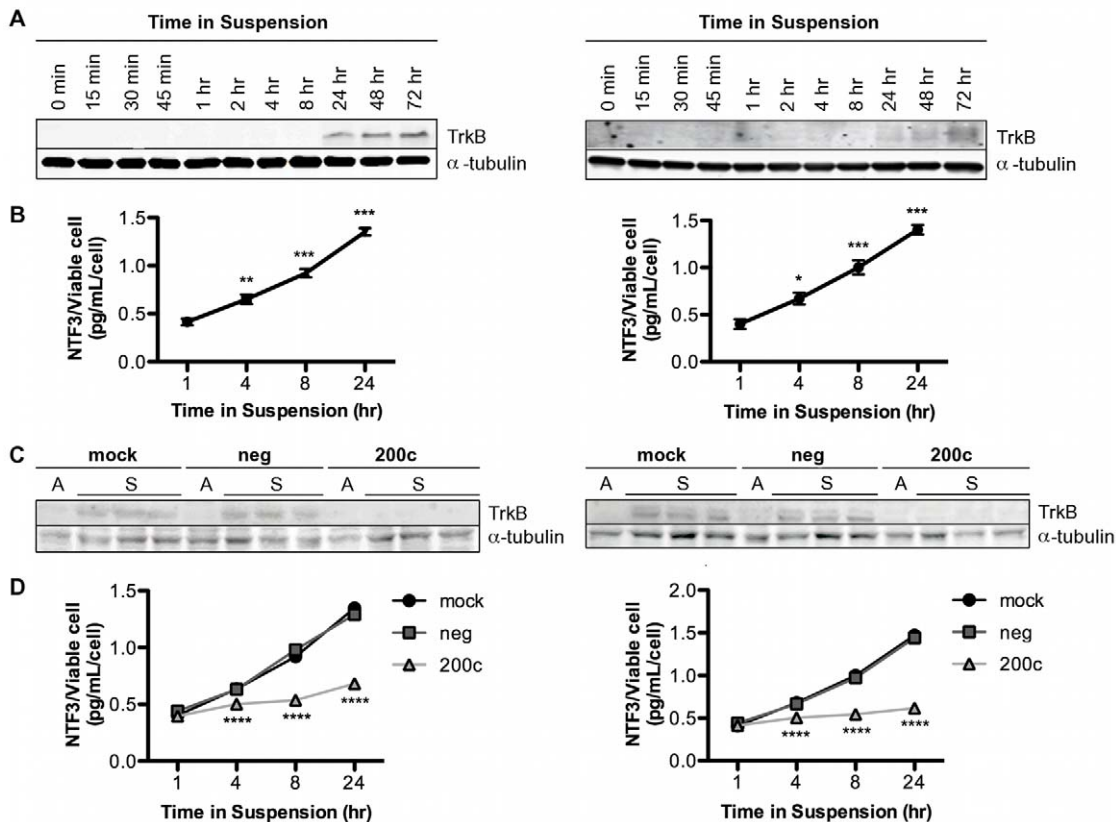
Figure 5. TrkB and NTF3 are up-regulated in suspended cells and miR-200c blocks this up-regulation. Cells were plated in suspension and harvested at the time points indicated. **A.** Immunoblot for TrkB expression, α-tubulin used as loading control. **B.** NTF3 ELISA performed on medium. *Points,* mean of three biological replicates, *bars,* SEM. Cells treated with transfection reagent only (mock), scrambled negative control (neg) or miR-200c mimic (200c) and 24 hrs later plated in suspension. **C.** Cells were harvested 24 hrs later and immunoblot performed for TrkB, α-tubulin used as loading control. **D.** NTF3 ELISA performed on medium at time points indicated. *Points,* mean of three biological replicates, *bars,* SEM.
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expressing mutant IκBα exhibit no decrease in IκBα expression, as expected (Fig. 7B). Furthermore, expression of mutant IκBα prevents up-regulation of NF-κB transcriptional activity, as indicated by luciferase reporter activity (Fig. 7C). Next we sought to determine if inhibition of NF-κB signaling would inhibit up-regulation of TrkB and NTF3, and found that mutant IκBα completely repressed up-regulation of both TrkB and NTF3 at the mRNA level (Fig. 7D). Similarly, TrkB was not up-regulated in mutant IκBα expressing suspended cells (Fig. 7E), nor was soluble NTF3 (Fig. 7F). Taken together this data shows that two NF-κB transcription factors bind directly to the TrkB and NTF3 promoters, and that activation of NF-κB transcriptional activity is required for suspension induced up-regulation of TrkB and NTF3. Importantly, mutant IκBα sensitizes BT549 and MDA-231 cells to anoikis (Fig. 7G), demonstrating the full affect of the pathway, from the necessity of NF-κB transcriptional activation to anoikis sensitivity. Thus, NF-κB transcriptionally up-regulates TrkB and NTF3 through direct binding of RelA and NF-κB1 to the regions in the promoters, and this increased transcription is necessary and sufficient for triple negative breast cancer cells to resist anoikis.

Discussion

Anoikis is not thought of as a classical component of EMT, but will perhaps soon be included since epithelial cells are sensitive to

anoikis, while mesenchymal cells are not [36–38]. Fibroblasts require loss of matrix attachment coupled with growth factor depletion to induce anoikis [39,40], while epithelial cells undergo anoikis even in the presence of serum. Additionally, resistance to anoikis is frequently observed in aggressive carcinoma cells, where it correlates with EMT [11,20–24]. Loss of the miR-200 family of miRNAs also correlates with EMT, and we show that expression of miR-200c correlates with resistance to anoikis in breast cancer cell lines (Fig. 1). Further, restoration of miR-200c to basal-like breast cancer cells restores sensitivity to anoikis (Fig. 1) and [11].

Although many miRNAs have been found to influence EMT and MET [41–43], few have been shown to affect anoikis. Various molecular mechanisms are employed to achieve anoikis resistance, and the mechanisms differ between cell types [44,45], complicating the identification of miRNA involvement. Hepatocellular carcinomas resist anoikis by expressing miR-221, which directly targets the pro-apoptotic protein Bmf [46]. Expression of miR-214 in melanoma cells promotes TFAP2C-mediated metastasis, mainly by promoting trans-endothelial migration, but also by suppressing anoikis resistance [47]. Finally, miR-451 suppresses anoikis resistance in non-small cell lung cancer [48], and miR-124 suppresses anoikis resistance in breast cancer [49], but molecular mechanisms (specific targets involved) remain to be identified. Our identification of TrkB [11] and NTF3 (Fig. 3) as direct targets responsible for the ability of miR-200c to restore anoikis sensitivity
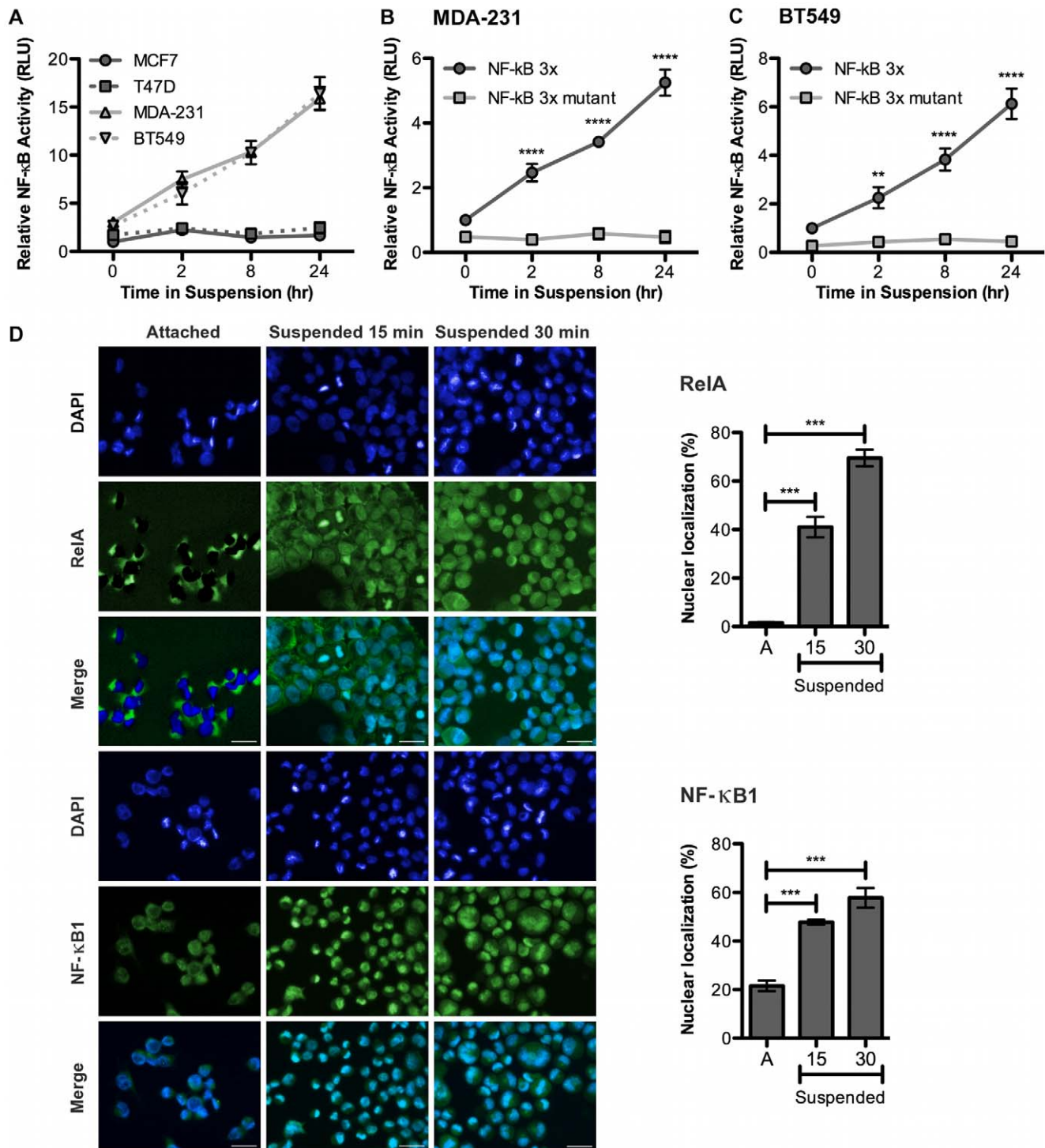
Figure 6. NF-κB transcriptional activity increases in suspended TNBC cells. A. Cells were transfected with 3x NF-κB transcriptional response element reporter and a *Renilla* control and 24 hrs later plated in suspension. Cells were harvested at time points indicated and dual luciferase assay performed. Data normalized to attached time point and presented relative to MCF7 attached condition. *Columns,* mean of three biological replicates, *bars,* SEM. MDA-231, **B**, and BT549, **C**, cells were transfected with 3x NF-κB or mutant reporter and assayed as in A. Data presented relative to NF-κB attached condition. *Columns,* mean of three biological replicates, *bars,* SEM. **D.** BT549 cells were grown on coverslips (attached), or in suspension and spun onto slides. Immunocytochemistry was performed for RelA or NF-κB1 (left), and the percentage of nuclear staining at each time point was quantitated (right). *Columns*, mean of three biological replicates, *bars*, SEM.
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establishes it as a prominent miRNA-mediator of anoikis sensitivity. Further, we demonstrate that TrkB signaling is both

necessary (Fig. 4), and sufficient (Fig. 2) for anoikis resistance in breast cancer. To our knowledge, this is also the first report of
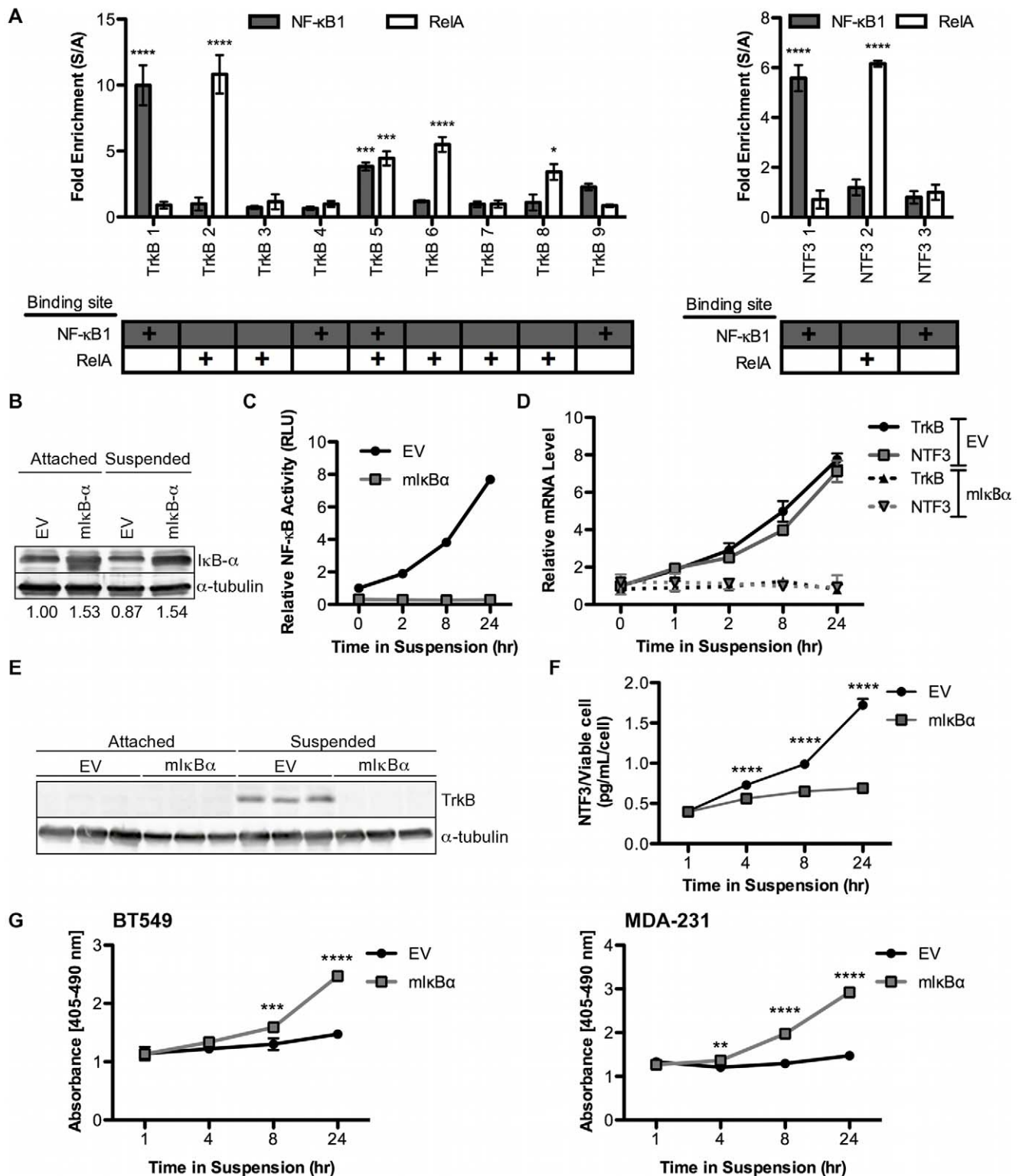
Figure 7. NF-κB transcriptionally up-regulates TrkB and NTF3 in suspended cells. A. BT549 cells were plated in suspension for 2 hrs and harvested for ChIP analysis. Following precipitation with antibodies against NF-κB1 and RelA, SYBR green qRT-PCR was performed for sites in the TrkB (left) and NTF3 (right) promoters. PLK1 used as a positive control for increased RelA binding in suspended cells. Data normalized to input controls and presented as a ratio of suspended over attached conditions. *Columns*, mean of three biological replicates, *bars*, SEM. **B–G** BT549 cells stably selected for empty vector (EV) or genetic NF-κB inhibition through mutant IκBα (mIκBα). **B.** Characterization of mIκBα cells, immunoblot of IκBα, α-tubulin used as loading control. Numbers represent amount of IκBα normalized to α-tubulin. **C.** Cells were transfected with 3×NF-κB transcriptional response element reporter and a *Renilla* control and 24 hrs later plated in suspension. Cells were harvested at time points indicated and dual luciferase assay performed. Data normalized to attached time point and presented relative to EV condition. *Points*, mean of three biological replicates, *bars*, standard error of the mean. **D.** Cells were plated in suspension and RNA was harvested at time points indicated. SYBR green qRT-PCR was performed for TrkB

and NTF3. Data normalized to actin and presented relative to attached. *Points*, mean of three biological replicates, *bars*, SEM. **E.** Cells were plated in suspension for 24 hrs and harvested for immunoblot analysis of TrkB, α-tubulin used as loading control. **F.** NTF3 ELISA performed on medium at time points indicated. *Points,* mean of three biological replicates, *bars*, SEM. **G.** BT549 (left) and MDA-231 (right) cells were plated in suspension and harvested at the time points indicated for analysis by Cell Death ELISA. *Points,* mean of three biological replicates, *bars*, SEM.
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a miRNA targeting both components of an autocrine signaling loop to protect against the aberrant expression of the receptor-ligand pair in an inappropriate cell type.

TrkB activated by exogenous BDNF confers resistance to anoikis [28–30,50], and induces EMT through transcriptional activation of several EMT-inducing transcription factors [21,22,32]. We present the first evidence that, in breast cancer, endogenous NTF3 is secreted upon suspension to enable TrkB-mediated anoikis resistance. Transformation with TrkB acts through Snail and Slug to induce ZEB1 transcription [21,22]. ZEB1 was recently found to be required for TrkB-induced EMT and anoikis resistance [22], and this likely due to the ability of ZEB1 to repress miR-200c [51,52]. We previously demonstrated that miR-200c directly targets TrkB [11] and, as we show here, NTF3 (Fig. 3). Figure 8 is a diagram depicting these established interactions and our new findings. Collectively, the data suggest that transformation with TrkB may lead to repression of the miR-200 family, the loss of which helps maintain the transformed state. However, in the basal-like TNBC cells used in this manuscript, miR-200c is already extremely low to absent compared to luminal A ER+breast cancer cells (Fig. 1 and [6]). Even though miR-200c is absent, in the attached state these cells do not express detectable TrkB protein, but we demonstrate that upon detachment TrkB and NTF3 are up-regulated. This response is dependent on increased active NF-κB driving transcription of both TrkB and NTF3 combined with the fact that miR-200c is not there to repress translation of the transcripts into protein. Further supporting an important role for loss of miR-200c in anoikis resistant breast cancers, grainyhead-like-2 (GRHL2) was recently reported to oppose EMT and anoikis resistance through direct repression of ZEB1 [53] (which results in an increase in miR-200c). GRHL2 is lost in more mesenchymal-like breast cancers, such as TNBC and claudin-low, suggesting that signaling converging on ZEB1 and miR-200c are important not just for EMT, but also for anoikis.

The NF-κB family of transcription factors is composed of two classes, Class I, containing NF-κB1 (p50/p105) and NF-κB2 (p52/p100), and Class II, containing RelA (p65), RelB (p68) and c-Rel (p75) [54,55]. NF-κB factors form hetero- or homodimers, which are held inactive in the cytoplasm. Thus, activation of NF-κB transcription requires only phosphorylation-induced degradation of the inhibitory IκB complex [56,57]. NF-κB is best known for its regulation of innate and adaptive immunity [58,59], where it was first discovered bound to the immunoglobulin promoter [60]. However, NF-κB also plays an important role in cancer, where it regulates proliferation and apoptosis [61,62]. Our findings uniquely demonstrate that, in anoikis resistant breast cancer cells, NF-κB transcriptional activity increases to mediate direct up-regulation of TrkB, and its ligand, NTF3 setting up an aberrant autocrine signaling loop. Axctivated Trk family members signal through Akt to facilitate cell survival [32,63–66]. Our finding that NF-κB up-regulates genes that facilitate anoikis resistance supports the appropriation of NF-κB signaling by cancer cells to avoid apoptosis. Specifically, our discovery of suspension-induced up-regulation of TrkB and NTF3 in breast cancer cells via NF-κB supports earlier work showing that polo-like kinase 1 (PLK1) is transcriptionally activated by RelA in suspended esophageal squamous cell carcinomas, leading to anoikis resistance [67].

Although few miRNAs have been implicated in anoikis, miR-125b has been found to be up-regulated in suspended mesenchymal stem cells, where it contributes to anoikis resistance through suppression of p53-mediated apoptosis [68]. This suggests that cells resist anoikis via different gene programs, depending on cell type of origin.

The mechanism by which NF-κB signaling is activated following loss of ECM attachment remains to be elucidated. However, integrin signaling is disrupted when the integrins are unligated to ECM components, yielding the possibility that integrin disruption activates NF-κB transcription. Integrins are obligate heterodimers, which link the ECM and the cytoskeleton. They are comprised of an α subunit, and a β subunit, and the specific α β composition dictates the ligand of the integrin [69]. Integrin-mediated activation of NF-κB signaling has been documented both in normal immune cell function, and in carcinoma models. However, the particular integrin mediating the activation, and the signaling program activated by NF-κB varies between systems. Neutrophils utilize α9β1 to avoid apoptosis through activated NF-κB signaling [70], while mono-cytes and monocyte-derived macrophages utilize αvβ3 to mediate a chronic inflammatory response [71]. In multiple myeloma, integrin β7 correlates with poor survival, activation of FAK, Src, and NF-κB signaling [72]. Various other integrin heterodimers have been found to activate NF-κB signaling in prostate cancer [73], melanoma [74], lung [75], and colorectal carcinoma models [76]. Interestingly, blockade of NF-κB signaling in gastric cancer prevents peritoneal dissemination of the disease through down-regulation of integrins α2, α3, and β1, which in turn prevents adhesion [77]. Future studies will determine if integrin signaling is responsible for the increased NF-κB induced upon detachment in breast cancer.

Finally, our identification of an autocrine signaling loop established by anoikis resistant breast cancer cells establishes a framework for exploration of combinatorial therapeutic strate-gies. Various strategies are employed by anoikis resistant cells, and many signal transduction pathways are concomitantly activated when cells should be committed to anoikis. Therefore, inhibition of multiple pathways should be the therapeutic aim, with a focus on avoiding activation of alternative pathways, and reducing toxicity. Because miR-200c targets genes involved not only in anoikis resistance [11], but in motility [8–12], proliferation [78,79], chemoresistance [80–82], and stemness [14,83], restora-tion of this miRNA along with an NF-κB inhibitor could serve as a potent combinatorial strategy.

Materials and Methods

Cell Culture and Treatments

MCF7 and T47D cells are available from the ATCC, and were grown in DMEM with 10% FBS, and 2 mM L-glutamine. MDA-231 cells are available from the ATCC, and were grown in MEM with 5% FBS, HEPES, NEAA, 2 mM L-glutamine, penicillin, streptomycin, and insulin. BT549 cells are available from the ATCC, and were grown in RPMI with 10% FBS and insulin. Hec50 cells were grown in DMEM with 10% FBS and 2 mM L-glutamine as described [84]. All cell line identities were verified by DNA profiling in the University of Colorado, DNA Sequencing
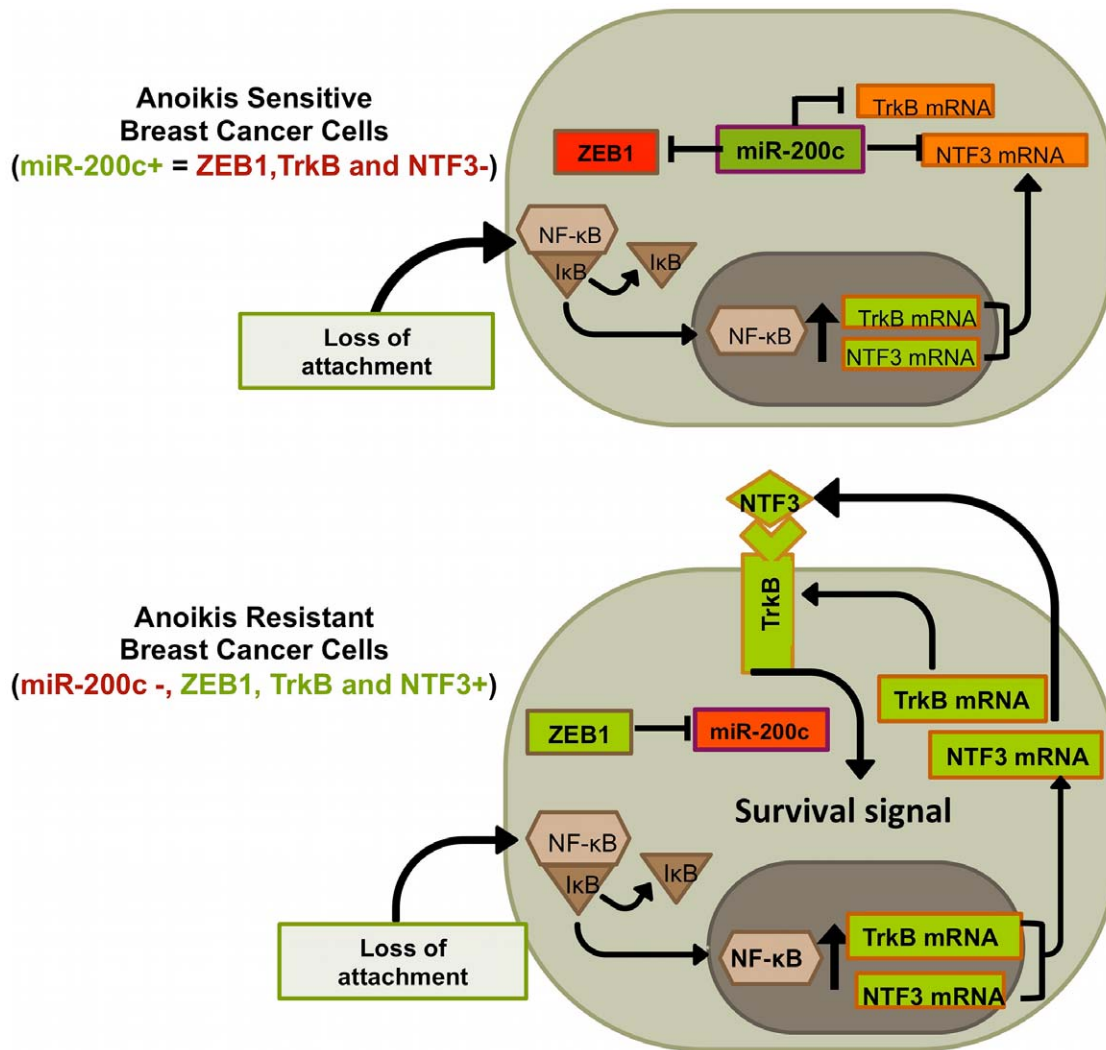
Figure 8. Model of select signaling pathways active in anoikis sensitive or resistant breast cancer cells. This model summarizes our findings regarding signaling pathways activated in breast cancer cells following loss of ECM attachment.
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and Analysis Core. Cells were treated with recombinant NTF3 (PeproTech, 450-03), and recombinant BDNF (PeproTech, 450-02) at the concentrations indicated.

Transfection and Transduction

TrkB was subcloned from pBabe-TrkB (a gift from D. Peeper, Netherlands Cancer Institute) into pcDNA3.1 (Invitrogen). The 3×NF-κB and mutant 3×NF-κB were gifts from A. Baldwin (University of North Carolina). mIκBα was generously provided by R. Schweppe (University of Colorado, AMC). All shRNA constructs are part of the Sigma-Aldrich MISSION line, obtained from the University of Colorado, Functional Genomics Core – shneg (SHC002), shTrkB (TRCN0000195114, TRCN0000002242) and shNTF3 (TRCN0000058853, TRCN0000058854). miR-200c mimic or scrambled negative control (Ambion) were transfected at a concentration of 50 nM. Plasmids were transfected according to the manufacturer's instructions. Lenti- and retroviral vectors were packaged in 293FT packaging cells (Invitrogen). All transfections were performed with Lipofectamine 2000 (Invitrogen) per the manufacturer's instructions.

Luciferase Assay

A section of the 3′ untranslated region (UTR) of NTF3 containing the putative binding sites for miR-200c was amplified by PCR from HeLa genomic DNA using the following primers, NTF3 F 5′ – CCACTAGTGCATGTAGCATA –3′, NTF3 R 5′ – CTCAAGCTTACAACAGTCAT –3′. Fragments were cloned into a firefly luciferase reporter vector (pMIR-REPORT, Ambion). Mutations were generated by PCR directed mutagenesis using the following primers, NTF3 mut1 F 5′ – TAAAATCTGTGTACACCATCTTTTTGC –3′, NTF3 mut1 R 5′ – TGACAAAGATGAATGCAAAATACTGGTG –3′, NTF3 mut2 F 5′ – TGCATTCATCTTTGTCAAGGCCAT-GACTGT –3′, NTF3 mut2 R 5′ – TGACAAAGATGAATG-CAAAATACTGGTG –3′. Luciferase assay was performed on Hec50 cells using the Dual Luciferase Reporter assay system (Promega, E1960). NF-κB luciferase assays were performed on cell lines indicated, using the same DLR kit. All luciferase measurements are normalized to *Renilla* readings.

Real-time Reverse Transcription-PCR

RNA was harvested from cells using Trizol (Invitrogen). SYBR Green real-time RT-PCR was performed using primers specific for each target, TrkB F 5′ – CCTGCTGGGGTAGTGGCTGCG – 3′, TrkB R 5′ – CATGGCATCCGTGTGGCCGT –3′, NTF3 F 5′ – CCTGCTGGGGTAGTGGCTGCG –3′, NTF3 R 5′ – CATGGCATCCGTGTGGCCGT –3′, ACTIN F 5′ – CTGTCCACCTTCCAGGAGATG –3′, ACTIN R 5′ – CGCAACTAAGTGATAGTCCGC –3′. To avoid the possibility of amplification artifacts, PCR products for all SYBR Green primer pairs were verified to produce single products by agarose electrophoresis and high resolution melt curve. The relative mRNA levels were calculated using the comparative Ct method (ΔΔCt).

Reagents

Primary antibodies used were TrkB (Cell Signaling, 4603S, 1:1000), IκBα (Santa Cruz, sc-847, 1:100), NF-κB1 (Abcam, ab7971, ChIP –2 µg/mL, ICC –1:150), RelA (Abcam, ab7970, ChIP –2 µg/mL, ICC –1:150), and α-tubulin (Sigma, clone B-5-1-2, 1:30,000). Goat anti rabbit conjugated to Alexa Fluor 660 (Invitrogen, 1:5000), and goat anti mouse conjugated to Alexa Fluor 660 (Invitrogen, 1:5000) were used as appropriate, and signal was detected by Odyssey Infrared Imaging System (Licor Biosciences). For ICC, goat anti rabbit conjugated to Alexa Fluor 488 (Invitrogen, 1:500) was used. NTF3 levels were detected by NTF3 ELISA (Promega, G7640).

Anoikis Assays (Cell Viability and Cell Death ELISA)

Poly-hydroxyethyl methacrylate (poly-HEMA, Sigma-Aldrich) was reconstituted in 95% ethanol to12 mg/mL, and used to coat plates. For DAPI/PI staining, cells were stained with DAPI (Sigma-Aldrich, D8417, 20 µg/mL) and PI (Sigma-Aldrich, P4170, 1 µg/mL). For cell death, cells were harvested and assayed by cell death ELISA (Roche, 1 920 685).

Digital Imaging

Images were collected using a Nikon ECLIPSE Ti system (Nikon). Quantitation was performed in ImageJ. Co-localization analysis was performed using the co-localization plug-in (http://rsbweb.nih.gov/ij/plugins/colocalization.html), which identifies pixels that exhibit fluorescence in both channels.

Chromatin Immunoprecipitation

BT549 cells were harvested following 2 hrs in suspension, cross-linked, and chromatin extracted as described [85]. Samples were sonicated for 10 seconds 8 times on a Branson 250 Sonicator (Emerson). qRT-PCR was performed as described above using the following primers: NTF3 CHIP F1 5′ – gaaaagcagaacccgacaga – 3′, NTF3 CHIP R1 5′ – cgcaagggtagggtagtcct –3′, NTF3 CHIP F2 5′ – cagggaggaaacgggatact –3′, NTF3 CHIP R2 5′ – agcagagttttgcccacttg –3′, NTF3 CHIP F3 5′ – acacacagcccctccc-tagt –3′, NTF3 CHIP R3 5′ – tagacccttccagctccaga –3′, TrkB CHIP F1 5′ – tgggtgattacgcacacact –3′, TrkB CHIP R1 5′ – ctgagctgcgcctctattct –3′, TrkB CHIP F2 5′ – agagccctcggaagtgtcag –3′, TrkB CHIP R2 5′ – tcctttaacctgacgggatg –3′, TrkB CHIP F3 5′ – gtgtgtgaactcccacatgc –3′, TrkB CHIP R3 5′ – caaaaacaca-cacacgctca –3′, TrkB CHIP F4 5′ – ggtgagcagcgcagatagt –3′, TrkB CHIP R4 5′ – taaaggggaatgcggagact –3′, TrkB CHIP F5 5′ – gaccagctcagcctctgata –3′, TrkB CHIP R5 5′ – catgccacctttatc-caggac –3′, TrkB CHIP F6 5′ – aaagtgctgtgtgtatgttgtgtt –3′, TrkB CHIP R6 5′ – ggatgccatctcctaagcaa –3′, TrkB CHIP F7 5′ – gttgaaatgcactcgctcaa –3′, TrkB CHIP R7 5′ – caatgctaaagc-

cagccttc –3′, TrkB CHIP F8 5′ – tgccaacgtagttgaccaag –3′, TrkB CHIP R8 5′ – atcctagcaccctggactca –3′, TrkB CHIP F9 5′ – tccaaagtctgtggcctttt –3′, TrkB CHIP R9 5′ – ccaccacacacacacaa-caa –3′, PLK1 CHIP F 5′ – ccgtgtcaatcaggttttcc –3′, PLK1 CHIP R 5′ – cgtcctcgtccgctcaccat –3′.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5. Student's t-test, ANOVA with Tukey post-hoc test, and two-way ANOVA with Bonferroni multiple comparison test were used as appropriate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$, NS – not significant.

Supporting Information

Figure S1 TrkB signaling does not affect survival in attached cells. MCF7 (top) and T47D (bottom) cells stably selected for expression of empty vector (EV) or TrkB were plated attached in increasing concentrations of BDNF or NTF3. Cells were harvested 24 hrs later and apoptosis assayed by Cell Death ELISA, data shown relative to EV conditions. *Columns,* mean of three biological replicates, *bars,* SEM.
(TIFF)

Figure S2 TrkB and NTF3 are required for anoikis resistance. MDA-231 cells stably selected for expression of shneg, shTrkB or shNTF3 constructs. **A.** Efficacy of TrkB knockdown. *Left,* immunoblot showing knockdown of TrkB, α-tubulin used as loading control, *right,* quantitation of immunoblot. **B.** Efficacy of NTF3 knockdown. NTF3 ELISA performed on medium. *Columns,* mean of three biological replicates, *bars,* SEM. **C.** Cell death ELISA performed on cells suspended for 24 hrs. *Columns,* mean of three biological replicates, *bars,* SEM. **D-G.** Cells treated with transfection reagent only (mock), scrambled negative control (neg) or miR-200c mimic (200c) and 24 hrs later plated in suspension. Cells were harvested 24 hrs later for analysis. **D.** Immunoblot for TrkB, α-tubulin used as loading control. **E.** NTF3 ELISA performed on medium. *Columns,* mean of three biological replicates, *bars,* SEM. shTrkB, **F,** and shNTF3, **G,** cells analyzed by cell death ELISA. *Columns,* mean of three biological replicates, *bars,* SEM.
(TIFF)

Figure S3 TrkB and NTF3 up-regulation is transcriptional. Cells were plated in suspension and RNA was harvested at time points indicated. SYBR green qRT-PCR was performed for TrkB and NTF3. Data normalized to actin and presented relative to attached time point. *Points,* mean of three biological replicates, *bars,* SEM.
(TIFF)

Acknowledgments

Author Contributions

Conceived and designed the experiments: ENH DRC DMC. Performed the experiments: ENH. Analyzed the data: ENH DRC DMC. Contributed reagents/materials/analysis tools: ENH DMC. Wrote the paper: ENH. Edited and approved the final manuscript: ENH DRC DMC JKR.

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Breast Cancer
R E S E A R C H

RESEARCH ARTICLE

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Targets of miR-200c mediate suppression of cell motility and anoikis resistance

Erin N Howe[1], Dawn R Cochrane[2] and Jennifer K Richer[1*]

Abstract

Introduction: miR-200c and other members of the miR-200 family promote epithelial identity by directly targeting ZEB1 and ZEB2, which repress E-cadherin and other genes involved in polarity. Loss of miR-200c is often observed in carcinoma cells that have undergone epithelial to mesenchymal transition (EMT). Restoration of miR-200c to such cells leads to a reduction in stem cell-like characteristics, reduced migration and invasion, and increased sensitivity to taxanes. Here we investigate the functional role of novel targets of miR-200c in the aggressive behavior of breast and endometrial cancer cells.

Methods: Putative target genes of miR-200c identified by microarray profiling were validated as direct targets using dual luciferase reporter assays. Following restoration of miR-200c to triple negative breast cancer and type 2 endometrial cancer cell lines that had undergone EMT, levels of endogenous target mRNA and respective protein products were measured. Migration and sensitivity to anoikis were determined using wound healing assays or cell-death ELISAs and viability assays respectively.

Results: We found that restoration of miR-200c suppresses anoikis resistance, a novel function for this influential miRNA. We identified novel targets of miR-200c, including genes encoding fibronectin 1 (FN1), moesin (MSN), neurotrophic tyrosine receptor kinase type 2 (NTRK2 or TrkB), leptin receptor (LEPR), and Rho GTPase activating protein 19 (ARHGAP19). These targets all encode proteins normally expressed in cells of mesenchymal or neuronal origin; however, in carcinoma cells that lack miR-200c they become aberrantly expressed and contribute to the EMT phenotype and aggressive behavior. We showed that these targets are inhibited upon restoration of miR-200c to aggressive breast and endometrial cancer cells. We demonstrated that inhibition of MSN and/or FN1 is sufficient to mediate the ability of miR-200c to suppress cell migration. Lastly, we showed that targeting of *TrkB* mediates the ability of miR-200c to restore anoikis sensitivity.

Conclusions: miR-200c maintains the epithelial phenotype not only by targeting ZEB1/2, which usually facilitates restoration of E-cadherin expression, but also by actively repressing a program of mesenchymal and neuronal genes involved in cell motility and anoikis resistance.

Introduction

Epithelial to mesenchymal transition (EMT) occurs during development as it is required for formation of the neural crest and palate, among other processes [1,2]. In cancer it is a pathological event associated with tumor progression and is thought to influence certain steps in the metastatic cascade, thereby contributing to the metastatic potential of carcinomas. Specifically, EMT likely contributes to the ability of carcinoma cells to invade through basement membrane and stroma and to intravasate into blood and lymph vessels [3-5]. The process of EMT is regulated by several transcription factors, including Twist, SNAIL, SLUG, ZEB1 (zinc finger E-box binding homeobox 1) and the closely related SIP1 (ZEB2), as reviewed in [6], which are transcriptional repressors of *E-cadherin*.

The miR-200 family of miRNAs, which includes miR-200c and miR-141 on chromosome 12 and miR-200a/b and miR-429 on chromosome 1, directly targets ZEB1 and ZEB2 [7-10]. Restoring miR-200c to aggressive breast, endometrial and ovarian cancer cells substantially

* Correspondence: jennifer.richer@ucdenver.edu
[1]Program in Cancer Biology, Department of Pathology, University of Colorado, Anschutz Medical Campus, Mail Stop 8104, P.O. Box 6511, Aurora, CO, USA
Full list of author information is available at the end of the article

decreases migration and invasion [9-13]. Since ZEB1 represses E-cadherin [14] and other genes involved in polarity [15], the reduction in migratory and invasive capacity observed when miR-200c is restored to cancer cells is widely thought to be due to the ability of miR-200c to target and repress ZEB1/2 which, in most cases, allows E-cadherin to be re-expressed. However, even in cell lines in which E-cadherin is not restored, miR-200c still dramatically reduces migration and invasion [11], implying that additional miR-200c targets can facilitate its ability to suppress cell motility.

We identify and confirm novel direct targets of miR-200c, including the genes encoding fibronectin 1 (FN1), moesin (MSN), neurotrophic tyrosine receptor kinase type 2 (NTRK2 or TrkB), leptin receptor (LEPR), and Rho GTPase activating protein 19 (ARHGAP19). These targets are all genes usually expressed in cells of mesenchymal or neuronal origin. However, in carcinoma cells that lack miR-200c, repression of these genes is compromised and they are allowed to be translated and contribute to an EMT phenotype and aggressive behavior. Here we show that *MSN* and *FN1* are direct targets of miR-200c that contribute to the ability of miR-200c to suppress migration. We also identify a completely novel role for miR-200c - the ability to reverse anoikis resistance and we further pinpoint *TrkB* as the direct target that mediates this effect. Anoikis resistance is an important, yet understudied, step in the metastatic cascade.

Materials and methods
Cell culture
Hec50 cells were cultured in DMEM with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. AN3CA cells and Ishikawa cells were grown in MEM with 5% FBS, nonessential amino acids (NEAA), penicillin, streptomycin and 1 nM insulin. MCF-7 cells were grown in DMEM with 10% FBS, and 2 mM L-glutamine. MDA-MB-231 cells were grown in MEM with 5% FBS, HEPES, NEAA, 2 mM L-glutamine, penicillin, streptomycin, and insulin. BT549 cells were grown in RPMI supplemented with 10% FBS and insulin. All cells were grown in a 37°C incubator with 5% CO_2. Cell line identities were authenticated by isolating genomic DNA using ZR genomic DNAII kit (Zymo Research, Irvine, CA, USA) and DNA profiling multiplex PCR was performed using the Identifiler Kit (Applied Biosystems, Carlsbad, CA, USA) in the UC Cancer Center DNA Sequencing and Analysis Core.

Transfection
miR-200c (miRNA mimic) or scrambled negative control (Ambion, Austin, TX, USA) at a concentration of 50 nM were incubated with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in culture medium per the

manufacturer's instructions before addition to cells. Cells were incubated at 37°C for 24 hrs before replacement of medium.

DNA and shRNA constructs
pEGP-MSN (created by Stephen Shaw, National Institutes of Health, purchased from Addgene plasmid 20671, Cambridge, MA, USA) [16]. *FN1* was subcloned from pCR-XL-TOPO-FN1 (Open Biosystems, Catalog number MHS4426-99240322, Huntsville, AL, USA) into pcDNA3.1 (Invitrogen). *TrkB* was subcloned from pBabe-TrkB (a gift from D. Peeper) into pcDNA3.1.

Microarray analysis
Expression profiling was performed on Hec50 cells transfected as described above and statistical analysis was performed as described previously [12]. Array data have been provided to GEO, accession GSE25332. The heatmap was generated using GeneSpring GX 11 (Agilent, Santa Clara, CA, USA) and shows genes that are statistically significantly down-regulated by at least 1.5-fold in the miR-200c treated samples as compared to either the mock or scrambled control or both, and are predicted to be direct targets of miR-200c. Target site predictions were taken from TargetScan [17], http://microRNA.org[18], PicTar [19] and MicroCosm [20].

Luciferase assays
A section of the 3' untranslated region (UTR) of each target containing the putative binding site(s) for miR-200c was amplified by PCR from HeLa genomic DNA using the primers listed in Table S1 in Additional file 1. Fragments were cloned into the 3' UTR of a firefly luciferase reporter vector (pMIR-REPORT, Ambion) using HindIII and SpeI. Mutations in the miR-200c binding sites were generated by PCR directed mutagenesis. Mutation primers are listed in Table S1 in Additional file 1 and introduced mutations are in bold and shown above the mRNA in each figure. 3' UTR sequences and mutations were verified by sequencing. Hec50 cells (15,000 per well) plated in a 96-well plate were mock transfected, transfected with negative control, 50 nM miR-200c, 50 nM miR-200c antagomiR (Dharmacon, Lafayette, CO, USA)) alone (α200c) or in conjunction with miR-200c (α200c + 200c). After 24 hrs, the firefly reporter plasmid (196 ng) and a *Renilla* luciferase normalization plasmid pRL-SV40 (4 ng) were introduced using Lipofectamine 2000. Cells were harvested 48 hrs later for analysis using the Dual Luciferase Reporter assay system (Promega, Madison, WI, USA)).

Real-time reverse transcription-PCR
RNA was harvested from cells using Trizol (Invitrogen) and treated with DNase 1 (Invitrogen) for 15 minutes at

room temperature. RNA was reverse transcribed into cDNA in a reaction containing reaction buffer, 10 mM DTT, 1 mM dNTPs, RNase inhibitor (Applied Biosystems), 250 ng random hexamers, and 200 units of MuLV-RT (Applied Biosystems). For normalization, real-time reverse transcription-PCR (RT-PCR) was performed on the cDNA using eukaryotic 18S rRNA endogenous control primers and FAM-MGB probe (Applied Biosystems). TaqMan MicroRNA Reverse Transcription kit was used to generate cDNA for real-time RT-PCR reaction in conjunction with a miR-200c specific primer and probe (Applied Biosystems, assay ID 002300). The reverse transcription primer for miR-200c is a hairpin primer specific to the mature miRNA and will not bind to the precursor molecules. For validation of the microarray data, SYBR Green real-time RT-PCR was performed using primers specific for each target (primers listed in Table S1 in Additional file 1). To avoid the possibility of amplification artifacts, PCR products for all SYBR Green primer pairs were verified to produce single products by agarose electrophoresis and high resolution melt curve. The relative mRNA or miRNA levels were calculated using the comparative Ct method ($\Delta\Delta$Ct). Briefly, the Ct (cycle threshold) values for the rRNA or actin were subtracted from Ct values of the target gene to achieve the ΔCt value. The $2^{-\Delta Ct}$ was calculated for each sample and then each of the values was divided by a control sample to achieve the relative mRNA or miRNA levels ($\Delta\Delta$Ct).

Immunoblot analysis

Whole-cell protein extracts prepared in RIPA lysis buffer, equalized to 50 µg by Bradford protein assay (Bio-Rad, Hercules, CA, USA), separated by SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes. For chemiluminecent detection, membranes were blocked in 5% milk in TBS-T and probed overnight at 4°C with primary antibodies. Primary antibodies used were ZEB1 (rabbit polyclonal from Dr. Doug Darling, University of Louisville, Louisville, KY, USA; 1:1,500 dilution), E-cadherin (clone NCH-38 from DAKO, Carpinteria, CA, USA; 1 µg/mL), fibronectin (BD Biosciences, Franklin Lakes, NJ, USA, clone 10/Fibronectin, 1:5000), moesin (Abcam, Cambridge, MA, USA, clone EP1863Y, 1:10,000), ERM (Cell Signaling, Danver, MA, USA, #3142, 1:1000), TrkB (Santa Cruz Biotechnology, Santa Cruz, CA, USA, H-181, #sc8316, 1:200) and α-tubulin (Sigma-Aldrich, St. Louis, MO, USA, clone B-5-1-2, 1:30,000). After incubation with appropriate secondary antibody, results were detected using Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer, Waltham, MA, USA). For fluorescent detection, membranes were blocked in 3% BSA (Sigma-Aldrich) in TBS-T and probed overnight at 4°C

with primary antibodies. Goat anti-rabbit conjugated to Alexa Fluor 660 (Invitrogen, 1:5,000) and goat anti mouse conjugated to Alexa Fluor 660 (Invitrogen, 1:5,000) were used as appropriate and signal was detected by Odyssey (LI-COR, Lincoln, NE, USA).

Wound healing assay

Cells were transfected with miR-200c and controls as before and 24 hrs later transfected with vectors. Cells were then plated in six-well plates, allowed to adhere and grow to confluency. Cells were then treated for two hours with 10 µg/mL mitomycin C (Fisher Scientific, Pittsburgh, PA, USA). Wounds were made using a p20 pipet tip and cells were given 24 hrs (Hec50 and BT549) or 48 hrs (AN3CA) to migrate into wounds. Cells were stained with 0.05% crystal violet in 6% glutaraldehyde for one hour, rinsed repeatedly with water, mounted and imaged. For each condition five representative images were obtained for quantitation. Quantitation was performed by first thresholding the images to differentiate between cells (black) and background (white), determining the number of black pixels and the number of white pixels and then calculating the percentage of the image covered by cells.

Anoikis assay (cell viability and cell death ELISA)

Poly-hydroxyethyl methacrylate (poly-HEMA, Sigma-Aldrich) was reconstituted in 95% ethanol to a concentration of 12 mg/mL. To prepare poly-HEMA coated plates, 0.5 mL of 12 mg/mL solution was added to each well of a 24-well plate and allowed to dry overnight in a laminar flow tissue culture hood. Cells were transfected as before. Twenty-four hours after transfection 50,000 cells were plated in triplicate in poly-HEMA coated 24-well plates using regular culture medium. For cell viability assay, at 4 and 24 hrs after addition to poly-HEMA coated plates, viable and dead cells were stained with trypan blue and counted using the ViCell cell counter (Beckman-Coulter, Brea, CA, USA). For cell death ELISA assay (Roche, San Francisco, CA, USA) cells were plated as before, but the medium was collected at 2, 4, 8, 24 and 48 hrs post plating. Each sample was pelleted, lysed and then frozen so that all samples could be read together at 405 nm and 490 nm (reference wavelength). The assay detects fragmented mono and oligonucleosomes in lysed cells by first binding histones with a biotinylated antibody which is bound to a streptavidin-coated plate. Samples are then bound by an HRP labeled anti-DNA antibody and color is developed by using an ABTS substrate.

Results

Restoration of miR-200c decreases non-epithelial, EMT associated genes

We utilize breast and endometrial cancer cell lines in which we have previously characterized miR-200c levels

as well as expression of classic epithelial and mesenchymal markers [11,12]. The BT549 and MDA-MB-231 cell lines are triple negative breast cancer (TNBC) cell lines, which lack expression of estrogen receptor alpha (ESR1), progesterone receptors, and HER2/neu. The TNBC lines lack E-cadherin and express the mesenchymal markers N-cadherin and vimentin and, therefore, exhibit an EMT phenotype. In contrast, MCF7 cells represent the luminal A subtype of breast cancer, which retains epithelial markers including ESR1 and E-cadherin. The Hec50 and AN3CA cell lines represent aggressive type 2 endometrial cancers that have lost epithelial markers including E-cadherin and ESR1 and gained mesenchymal markers such as N-cadherin and vimentin, indicative of EMT. In contrast, Ishikawa cells represent the less aggressive type 1 endometrial cancer, which retains epithelial markers and does not express mesenchymal markers. Transfection of miR-200c mimic into the dedifferentiated breast and endometrial cancer lines (BT549, MDA-MB-231, Hec50 and AN3CA) results in levels of mature miR-200c comparable to endogenous levels in the more well-differentiated breast and endometrial cancer lines (MCF7 and Ishikawa) (Figure 1a). These results indicate that experiments performed using this concentration of mimic result in miR-200c levels comparable to those observed in cell lines that have not undergone EMT.

By microarray expression profiling, we previously identified genes significantly altered upon restoration of miR-200c to Hec50 cells [12]. Figure 1b is a heatmap of genes known to be involved in EMT that are statistically significantly decreased at least 1.5-fold upon restoration of miR-200c and are bioinformatically predicted to be targets of miR-200c. The heatmap additionally depicts miR-200c targets identified by others such as *ZEB1* and 2 [8,9], cofilin (*CFL1*) [9] and *WAVE3* [21]. In total we identified 74 genes that change more than 1.5-fold and are predicted by two of four target prediction programs to be direct targets of miR-200c Figure S1 in Additional file 1. Of these genes, 68 (92%) are repressed and 6 (8%) are up-regulated when miR-200c is restored. Initial validation of several of the targets with known involvement
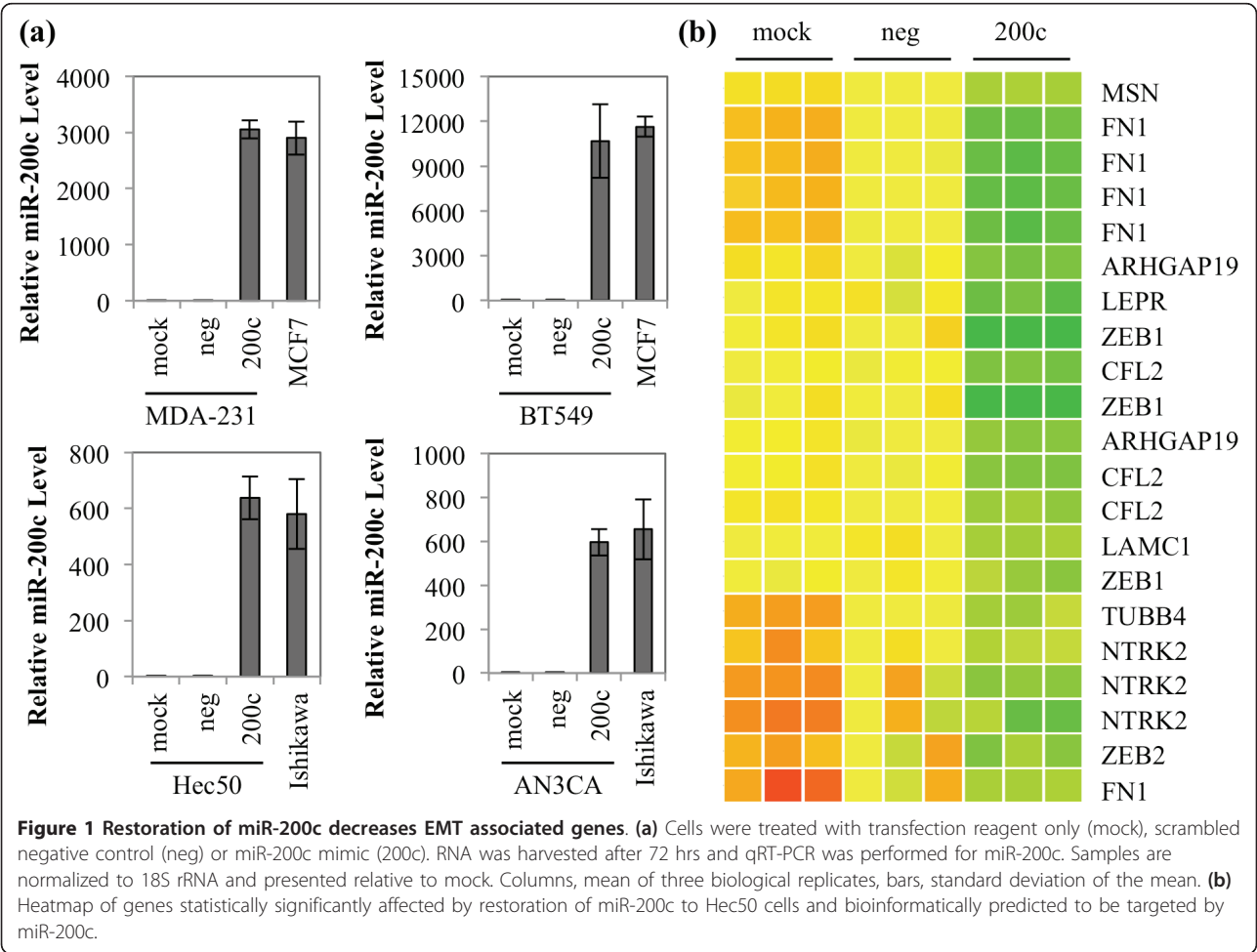

Figure 1 Restoration of miR-200c decreases EMT associated genes. **(a)** Cells were treated with transfection reagent only (mock), scrambled negative control (neg) or miR-200c mimic (200c). RNA was harvested after 72 hrs and qRT-PCR was performed for miR-200c. Samples are normalized to 18S rRNA and presented relative to mock. Columns, mean of three biological replicates, bars, standard deviation of the mean. **(b)** Heatmap of genes statistically significantly affected by restoration of miR-200c to Hec50 cells and bioinformatically predicted to be targeted by miR-200c.

in EMT revealed that they are down-regulated at the message level in one or more of our model cell lines Figure S2 in Additional file 1. Based on these findings, we selected *FN1, MSN, ARHGAP19, LEPR* and *TrkB* (*NTRK2* on the heatmap) to experimentally confirm as direct targets of miR-200c.

Breast and endometrial cancer cell lines that have undergone EMT and express ZEB1, also express FN1, MSN or both

Since there is substantial evidence in the literature for FN1 and MSN being involved in cancer cell migration, we assayed the breast and endometrial cancer cell lines for expression of these proteins (Figure 2). We found that neither the luminal A breast cancer cell line (MCF7) or the type 1 endometrial cancer cell line (Ishikawa) express FN1 or MSN, consistent with their pre-EMT phenotype, indicated by expression of E-cadherin and lack of ZEB1. In contrast, all of the TNBC and type 2 endometrial cancer lines express either one or both of these proteins in addition to ZEB1, supporting the hypothesis that they may play a role in migration in the absence of miR-200c.

Moesin (*MSN*), a regulator of cortical actin-membrane binding, is directly targeted and down-regulated by miR-200c

MSN connects the actin cytoskeleton and the cell membrane [22] and is strongly up-regulated in cancers with a poor prognosis, including metastatic breast cancer [23], where it contributes to migratory and invasive

capacity [24-26]. The 3' UTR of *MSN* contains two putative miR-200c binding sites (Figure 3a) and we cloned the region containing these sites downstream of luciferase. When miR-200c is restored, we observe a 37% decrease in luciferase activity only in the presence of miR-200c and not the controls. To determine the specificity of this down-regulation, we mutated the putative miR-200c binding sites and observe that luciferase activity levels return to levels observed in the absence of miR-200c; thus, miR-200c binding to these sites specifically is required for down-regulation. We also observe that mutating either binding site results in a partial increase in luciferase activity, but only when both sites are mutated is there a full restoration of luciferase activity. Therefore, both binding sites are functional and required for miR-200c to exert its full effect on the *MSN* 3' UTR. When an antagomiR is used to inhibit miR-200c binding to the target sites, luciferase activity is again restored. This indicates that miR-200c specifically is responsible for targeting the *MSN* 3' UTR and the consequent decrease in luciferase activity. Importantly, restoration of miR-200c decreases MSN protein levels (Figure 3b) in two cell lines that express detectable MSN protein, indicating that direct targeting of *MSN* by miR-200c exerts a measurable effect on MSN protein expression.
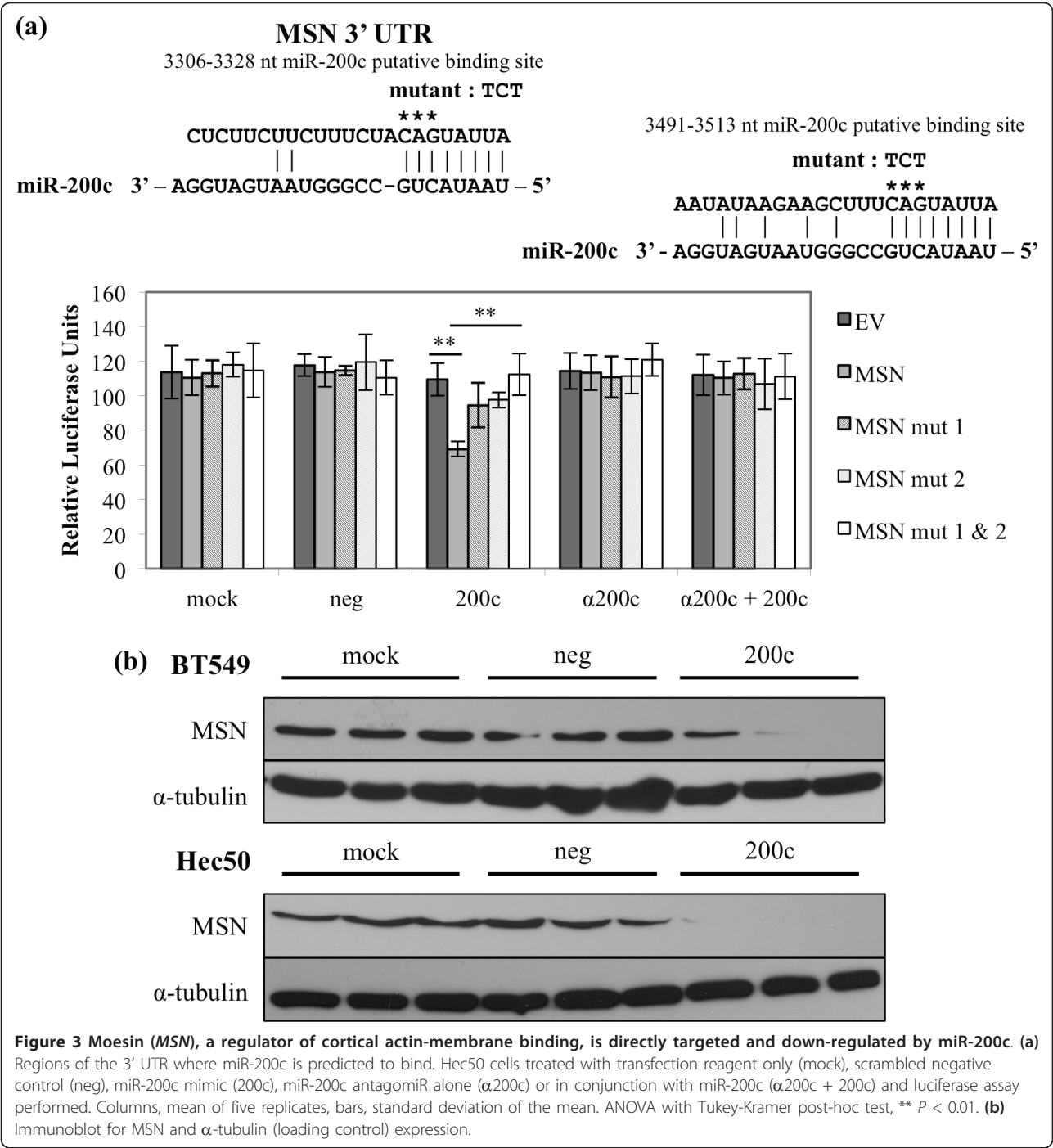
Down-regulation of MSN contributes to miR-200c mediated suppression of migration

Because miR-200c decreases migration, we next sought to determine the role of MSN in the ability of miR-200c to inhibit migration. Restoration of miR-200c to BT549 and Hec50 cells results in a dramatic decrease in their ability to close a wound as indicated by movement of cells past the initial boundary of the wound (black line) (Figure 4a). BT549 cells display a 41% decrease in migratory ability, while Hec50 cells display a 32% decrease (Figure 4b). The addition of a plasmid encoding MSN lacking its 3' UTR, rendering it untargetable by miR-200c, abolishes the ability of miR-200c to decrease migration (Figure 4a, b) without further increasing the migratory ability of the mock and negative control transfected cells. This indicates that miR-200c targeting of *MSN* can play a critical role in the ability of miR-200c to decrease migration in these cell lines. The levels of MSN protein achieved with the transfection are reasonable (Figure 4c) and do not interfere with the ability of miR-200c to restore E-cadherin in these cell lines.
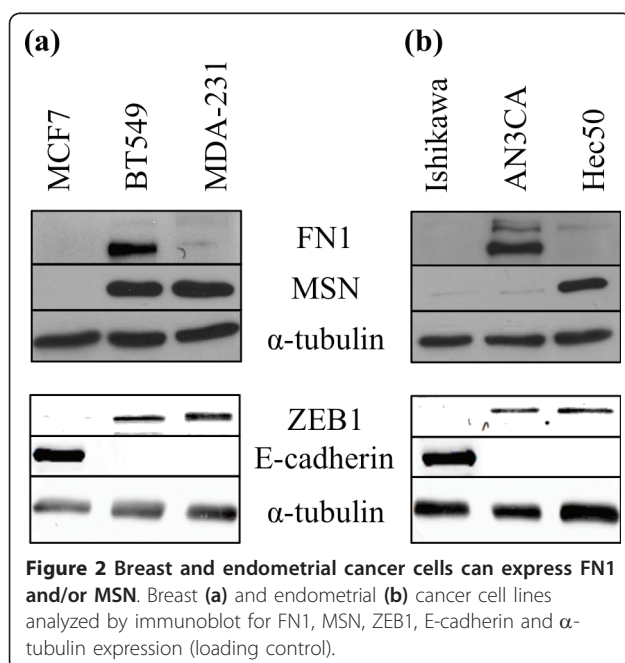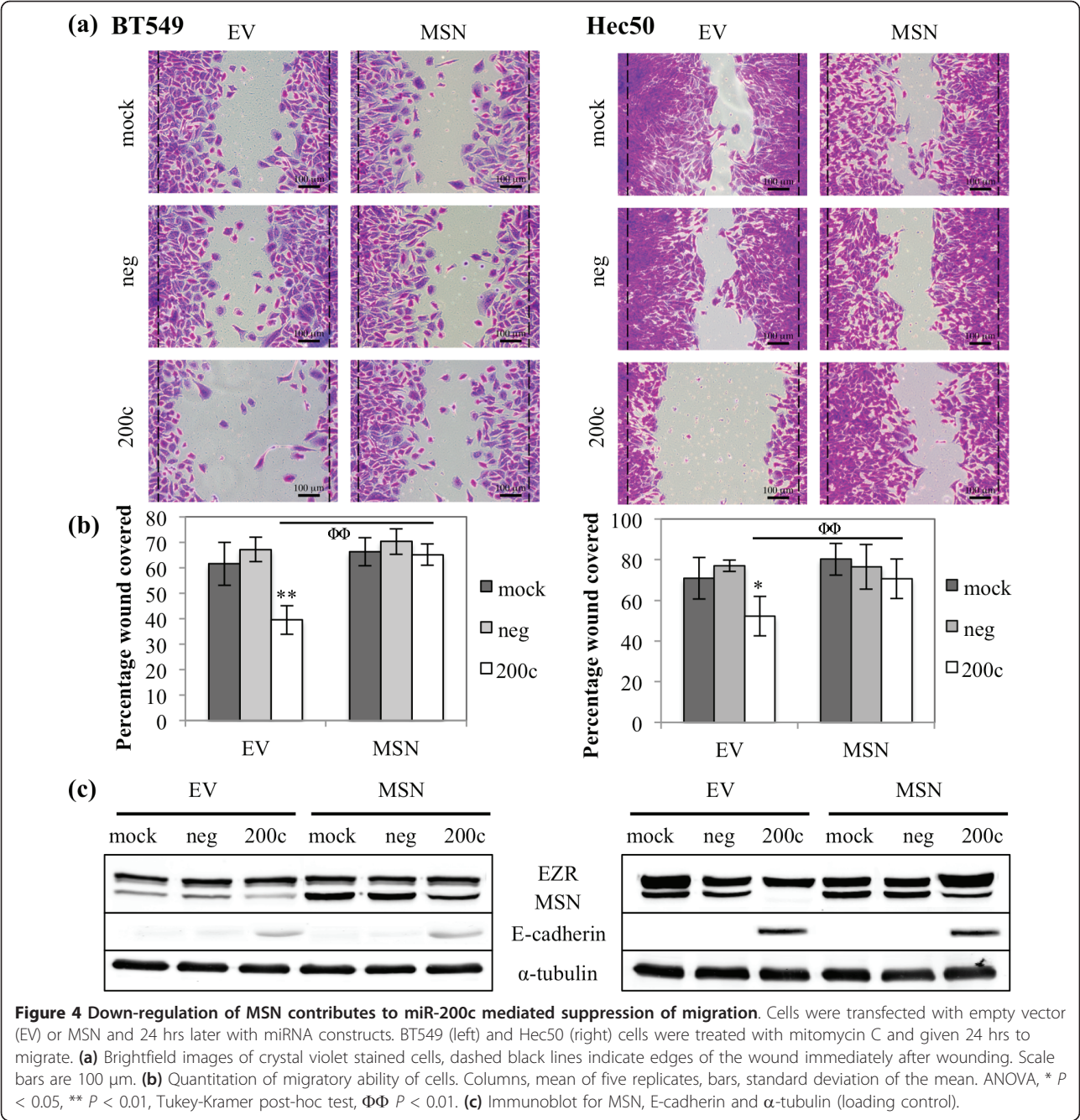
The extracellular matrix protein fibronectin 1 (*FN1*) is directly targeted and down-regulated by miR-200c

FN1 is normally expressed by fibroblasts but not epithelial cells, and is a classic marker of the EMT phenotype

Figure 2 Breast and endometrial cancer cells can express FN1 and/or MSN. Breast **(a)** and endometrial **(b)** cancer cell lines analyzed by immunoblot for FN1, MSN, ZEB1, E-cadherin and α-tubulin expression (loading control).

Figure 3 Moesin (*MSN*), a regulator of cortical actin-membrane binding, is directly targeted and down-regulated by miR-200c. (a)
Regions of the 3' UTR where miR-200c is predicted to bind. Hec50 cells treated with transfection reagent only (mock), scrambled negative control (neg), miR-200c mimic (200c), miR-200c antagomiR alone (α200c) or in conjunction with miR-200c (α200c + 200c) and luciferase assay performed. Columns, mean of five replicates, bars, standard deviation of the mean. ANOVA with Tukey-Kramer post-hoc test, ** $P < 0.01$. **(b)** Immunoblot for MSN and α-tubulin (loading control) expression.

and tumorigenicity [27-29]. We [12] and others [8] previously observed a decrease in *FN1* transcript upon restoration of miR-200c and we sought to determine if this is due to direct targeting. Like *MSN*, *FN1* contains two putative miR-200c binding sites in its 3' UTR. When miR-200c is restored, we observe a 76% decrease in luciferase activity only in the presence of miR-200c and not in the controls (Figure 5a). As for *MSN*,

mutated constructs show that miR-200c binding to these sites specifically is required for down-regulation and both binding sites are functional and required for miR-200c to exert its full effect on the *FN1* 3' UTR. When an antagomiR is used to inhibit miR-200c binding to the target sites, luciferase activity is again restored. This indicates that miR-200c specifically is responsible for targeting the *FN1* 3' UTR and the consequent

Figure 4 Down-regulation of MSN contributes to miR-200c mediated suppression of migration. Cells were transfected with empty vector (EV) or MSN and 24 hrs later with miRNA constructs. BT549 (left) and Hec50 (right) cells were treated with mitomycin C and given 24 hrs to migrate. **(a)** Brightfield images of crystal violet stained cells, dashed black lines indicate edges of the wound immediately after wounding. Scale bars are 100 μm. **(b)** Quantitation of migratory ability of cells. Columns, mean of five replicates, bars, standard deviation of the mean. ANOVA, * P < 0.05, ** P < 0.01, Tukey-Kramer post-hoc test, ΦΦ P < 0.01. **(c)** Immunoblot for MSN, E-cadherin and α-tubulin (loading control).

decrease in luciferase activity. Only the AN3CA and BT549 express detectable protein levels (Figure 2) and restoration of miR-200c to these cell lines dramatically decreases FN1 protein expression (Figure 5b).

Down-regulation of FN1 contributes to miR-200c mediated suppression of migration

We next sought to determine if FN1 plays a role in miR-200c control of migration. Restoration of miR-200c to BT549 and AN3CA cells again results in a dramatic

decrease in migration (Figure 6a), which is abrogated by addition of an untargetable *FN1* plasmid. The BT549 cells exhibit a 43% decrease in migratory ability, while the AN3CA cells decrease 53% (Figure 6b). Thus, down regulation of FN1 is an additional mechanism by which miR-200c suppresses migration in aggressive breast and endometrial cancer cell lines. The levels of FN1 protein achieved with the plasmid are reasonable and do not interfere with the ability of miR-200c to restore E-cadherin expression in the BT549 cell (Figure 6c). The
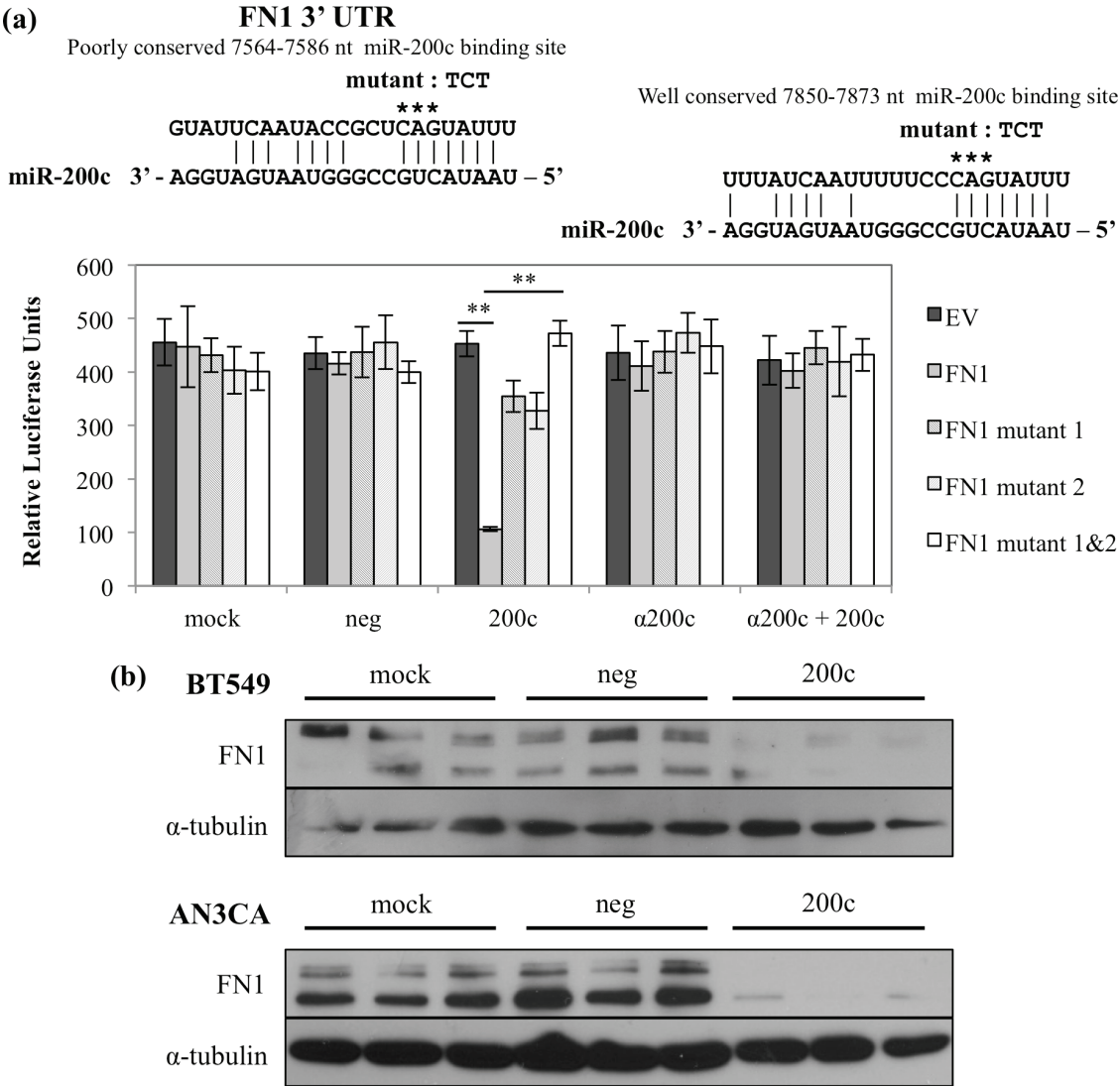
Figure 5 The extracellular matrix protein fibronectin (*FN1*) is directly targeted and down-regulated by miR-200c. (a) Regions of the 3' UTR where miR-200c is predicted to bind. Hec50 cells treated and luciferase assay performed. Columns, mean of five replicates, bars, standard deviation of the mean. ANOVA with Tukey-Kramer *post-hoc* test, ** $P < 0.01$. **(b)** Immunoblot for FN1 and α-tubulin (loading control) expression.
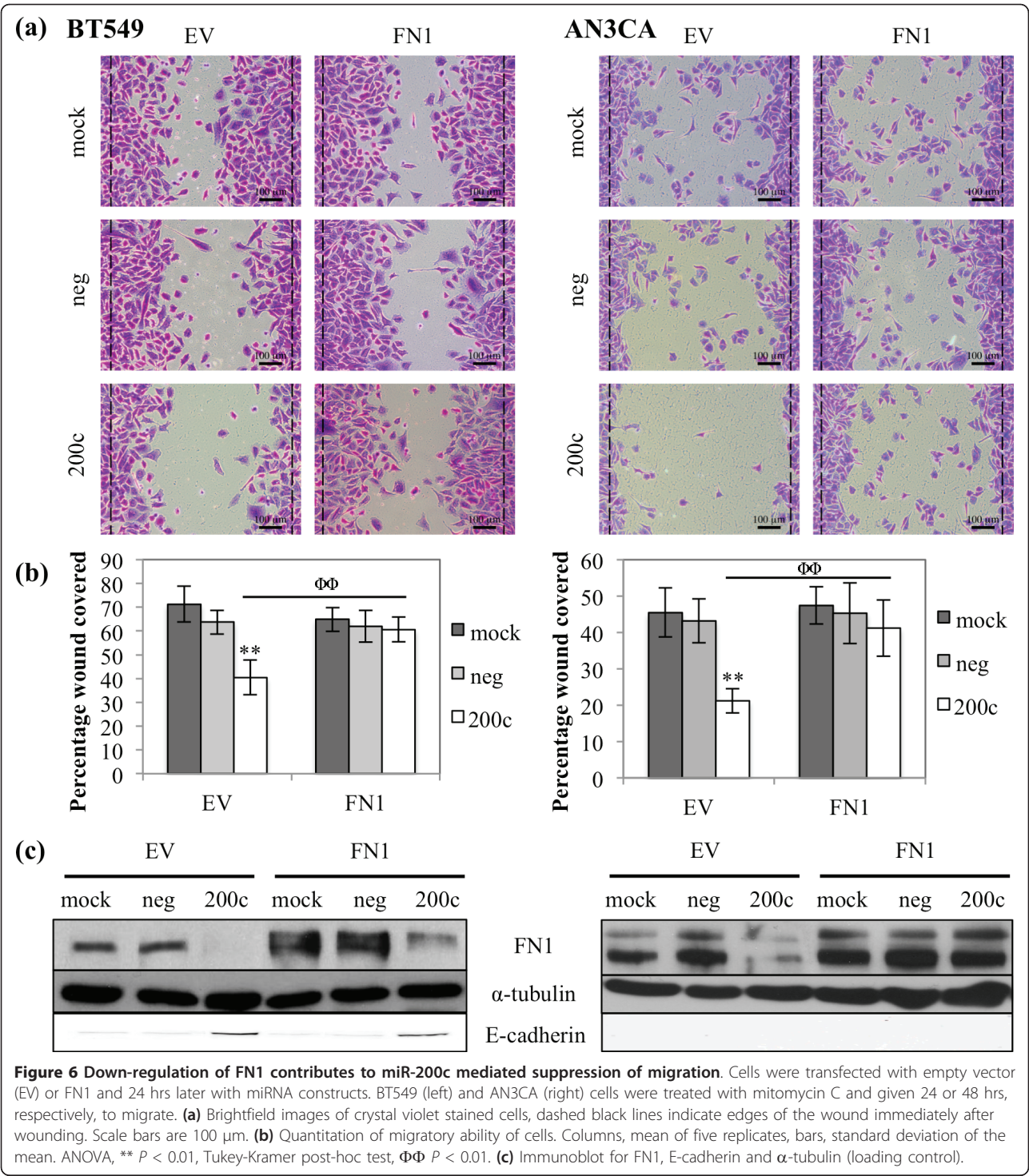
AN3CA cells do not re-express E-cadherin following restoration of miR-200c.

The genes encoding Rho GTPase activating protein 19 (*ARHGAP19*) and leptin receptor (*LEPR*) are directly targeted and down-regulated by miR-200c

ARHGAP19 is a GTPase activating protein that has not been well characterized, but is predicted to regulate the activity of Cdc42, RhoA and/or Rac1 [30]. The 3' UTR of *ARHGAP19* contains one putative miR-200c binding site. We demonstrate that restoration of miR-200c causes an 80% reduction in luciferase activity only in the presence of miR-200c and not in the controls (Figure S3 in Additional file 1). LEPR and its ligand leptin are

involved in the migration/invasion of trophoblasts [31] and the expression of leptin by mammary epithelial cells has been linked to tumorigenicity [32-34]. We demonstrate that restoration of miR-200c causes a 36% reduction in luciferase activity when the 3' UTR of *LEPR* is placed downstream of luciferase (Figure S4 in Additional file 1).

The anoikis suppressing neurotrophic receptor tyrosine kinase 2 (*NTRK2* or *TrkB*) is directly targeted and down-regulated by miR-200c

TrkB expression leads to anoikis resistance in several types of cancer, including breast [35-38], and this led us to investigate the regulation of this cell surface receptor
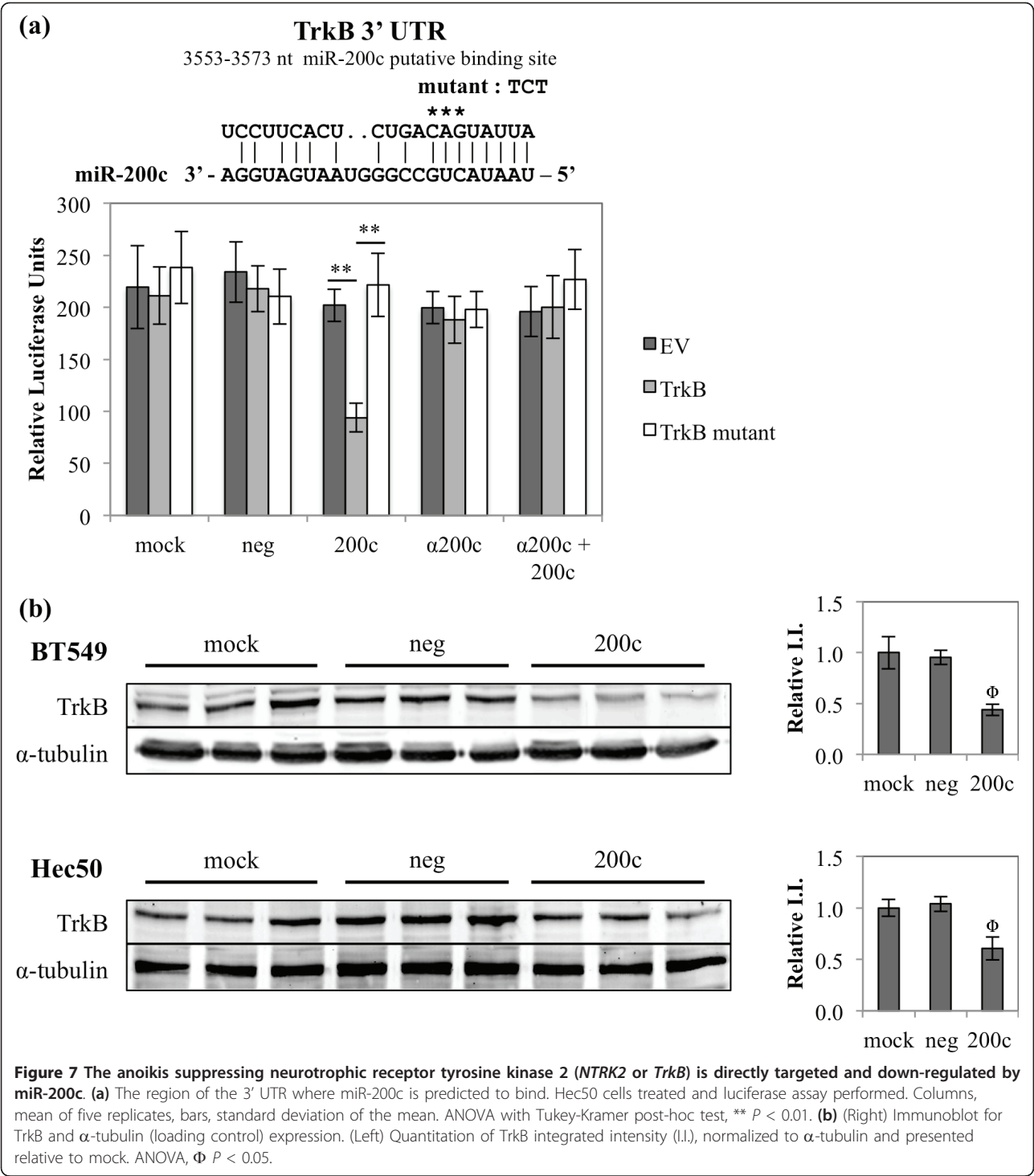
Figure 6 Down-regulation of FN1 contributes to miR-200c mediated suppression of migration. Cells were transfected with empty vector (EV) or FN1 and 24 hrs later with miRNA constructs. BT549 (left) and AN3CA (right) cells were treated with mitomycin C and given 24 or 48 hrs, respectively, to migrate. **(a)** Brightfield images of crystal violet stained cells, dashed black lines indicate edges of the wound immediately after wounding. Scale bars are 100 μm. **(b)** Quantitation of migratory ability of cells. Columns, mean of five replicates, bars, standard deviation of the mean. ANOVA, ** $P < 0.01$, Tukey-Kramer post-hoc test, ΦΦ $P < 0.01$. **(c)** Immunoblot for FN1, E-cadherin and α-tubulin (loading control).

by miR-200c. We demonstrate that *TrkB* is a direct target of miR-200c, showing a 55% reduction in luciferase activity (Figure 7a). Luciferase activity is restored following either mutation of the binding site or addition of an antagomiR, indicating that miR-200c binds to the 3' UTR of *TrkB* to downregulate it. Additionally, restoration of

miR-200c significantly decreases endogenous TrkB protein in the BT549 and Hec50 cells (Figure 7b).

miR-200c suppresses anoikis resistance
Given the known role of TrkB in anoikis resistance, we investigated the effect of miR-200c on anoikis by

Figure 7 The anoikis suppressing neurotrophic receptor tyrosine kinase 2 (*NTRK2* or *TrkB*) is directly targeted and down-regulated by miR-200c. **(a)** The region of the 3' UTR where miR-200c is predicted to bind. Hec50 cells treated and luciferase assay performed. Columns, mean of five replicates, bars, standard deviation of the mean. ANOVA with Tukey-Kramer post-hoc test, ** $P < 0.01$. **(b)** (Right) Immunoblot for TrkB and α-tubulin (loading control) expression. (Left) Quantitation of TrkB integrated intensity (I.I.), normalized to α-tubulin and presented relative to mock. ANOVA, Φ $P < 0.05$.

performing cell viability assays and cell death ELISAs. In these assays the cells are plated on poly-HEMA coated plates, which prevents them from adhering. The cells are forced to float in suspension for the times indicated before being harvested for analysis. Cell viability was determined by trypan blue exclusion and shows that restoration of miR-200c significantly decreases viability as quickly as 24 hrs in suspension (Figure 8a). In the cell death ELISAs, restoration of miR-200c results in an increase in fragmented nucleosomes, indicating an increase in apoptosis in these samples (Figure 8b). Thus, restoration of miR-200c decreases anoikis resistance as
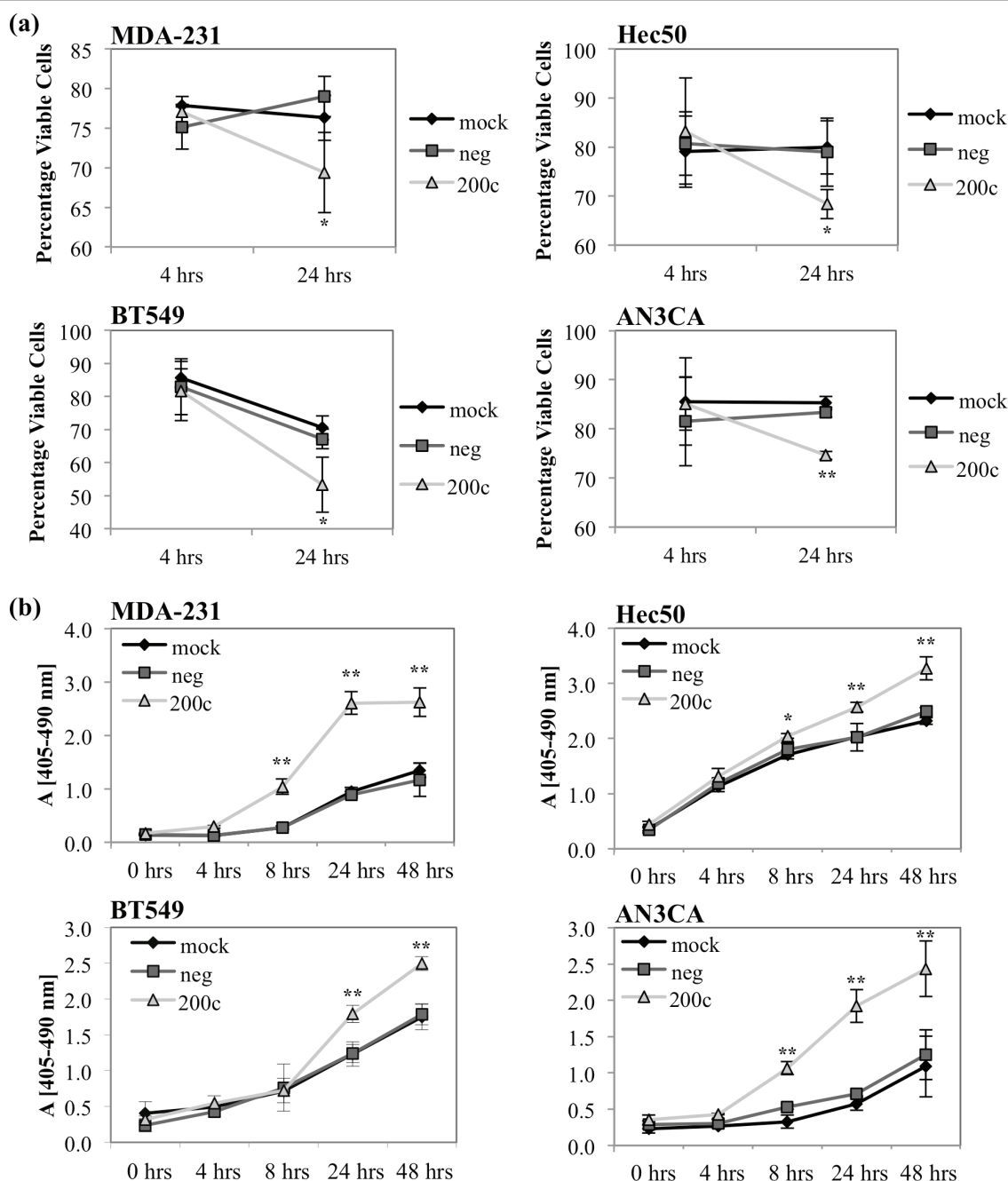
Figure 8 miR-200c increases sensitivity to anoikis. Breast (left) and endometrial (right) cancer cells were transfected with miRNA constructs and plated on poly-HEMA coated plates. Cells were collected for viability analysis by trypan blue exclusion **(a)** or apoptosis analysis by cell death ELISA **(b)**. Columns, mean of three biological replicates, bars, standard deviation of the mean. ANOVA, * $P < 0.05$, ** $P < 0.01$.

indicated by a decrease in the viability of suspended cells and concurrent increase in apoptosis.

Down-regulation of TrkB contributes to miR-200c mediated suppression of anoikis resistance

To determine if targeting of *TrkB* is responsible for the ability of miR-200c to restore sensitivity to anoikis, we used a plasmid encoding *TrkB* lacking the 3' UTR, rendering it untargetable by miR-200c. Restoration of miR-200c enhances sensitivity to anoikis (Figures 8 and 9), but this phenotype is completely reversed in the presence of exogenous, untargetable *TrkB* (Figure 9a, c). However, it is important to note that the addition of exogenous TrkB does not decrease the amount of cell
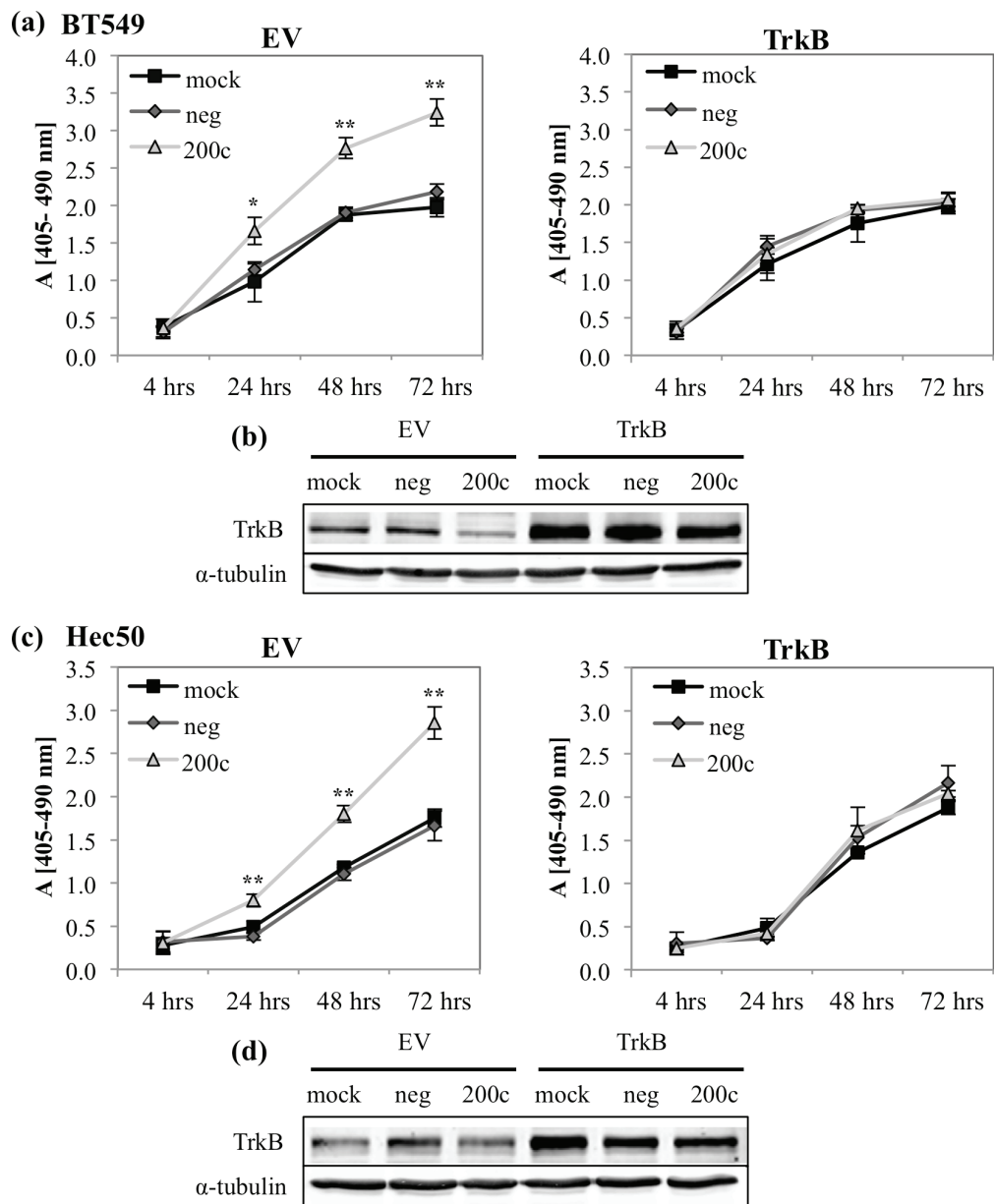
Figure 9 Down-regulation of TrkB contributes to miR-200c mediated suppression of anoikis resistance. Cells were transfected with empty vector (EV) (left) or TrkB (right) and 24 hrs with miRNA constructs. Twenty-four hours later cells were plated on poly-HEMA coated plates and cell death ELISA performed at time points indicated **(a)** and **(c)**. Columns, mean of three biological replicates, bars, standard deviation of the mean. ANOVA, * $P < 0.05$, ** $P < 0.01$. **(b)** and **(d)** Immunoblot for TrkB and α-tubulin (loading control).

death in mock or negative control transfected cells. This indicates that miR-200c targeting of *TrkB* plays a critical role in the ability of miR-200c to reverse anoikis resistance.

Discussion

Progression and metastasis of carcinomas is a multistep process. EMT is thought to aid cancer cells as they invade through basement membrane and stroma,

intravasate into blood or lymph vessels, and may also facilitate anoikis resistance, allowing tumor cells to survive the journey to the metastatic site. We sought to identify additional direct targets of miR-200c that mediate its potent effects.

Three of the new direct targets of miR-200c that we identify, *MSN*, *FN1*, and *ARHGAP19*, have been implicated in migration and invasion. MSN localizes to the trailing edge of invasive melanoma cells and disruption

of this localization leads to decreased metastasis [25]. MSN expression correlates with poor prognosis in oral squamous cell carcinoma [24] and basal breast cancer [23], a subtype with high risk of metastasis and recurrence. FN1 functions in cell migration through integrin binding [39] and can activate focal adhesion kinase (FAK) leading to increased motility and invasion of carcinoma cells [27,28]. ARHGAP19 is a member of a family of GTPase activating proteins, and other family members, 8, 9, 12 and 15, are expressed in several types of cancer and activate Cdc42, Rac1 or RhoA [40-43], small GTPases required for migration. We demonstrate that *FN1* and *MSN* are, at least in some cell lines, critical targets sufficient to mediate miR-200c's ability to inhibit migration in an *in vitro* wound healing assay. In some cell lines both MSN and FN1 are expressed, and in those cells both MSN and FN1 may contribute to migratory potential, but they are both repressed when miR-200c is restored. In other TNBC cells and type 2 endometrial cancer cells, either MSN or FN1 are expressed but not both. It is possible that even though miR-200c is absent, additional miRNA(s) that target these genes may be retained in some cells, or alternatively, factors that induce these genes at the promoter may be differentially expressed. In some cases ARHGAP19 may additionally contribute to migratory capacity; however, at present there is no antibody available to detect this protein. Loss of miR-200c could permit any of these genes, typically expressed in the more motile mesenchymal or neuronal cell types, to be inappropriately translated and expressed in epithelial cells. Expression of proteins such as MSN that actively contribute to cell motility by promoting front-rear polarity, combined with the loss of E-cadherin (which would decrease cell-cell attachments and reduce apical-basal polarity), may significantly contribute to the invasive capacity of carcinomas.

We demonstrate that restoration of miR-200c leads to a dramatic increase in sensitivity to anoikis (over a 100% increase in anoikis in some cell lines) and identify *TrkB* as a novel direct target of miR-200c. TrkB is a tyrosine kinase cell surface receptor typically expressed on neurons, which can be inappropriately expressed in carcinomas [44]. In breast and ovarian cancer cell lines TrkB induces anoikis resistance [31,33] and can induce EMT through activation of Twist [41]. We previously demonstrated that miR-200c does not affect apoptosis when endometrial cancer cells are attached to plastic, although it does enhance apoptosis induced by taxanes [11,12]. Thus, we conclude that miR-200c specifically enhances anoikis sensitivity, suggesting that restoration of miR-200c could limit the ability of breast and endometrial cancer cells to survive in the bloodstream.

Interestingly, all of the new miR-200c direct targets that we identify in this study (as well as other previously identified targets such as ZEB1/2 and TUBB3) contribute to the designation of this miRNA as a "guardian of the epithelial phenotype" because they are genes typically expressed in cells of mesenchymal or neuronal origin, but not in normal, well-differentiated epithelial cells.

Not all of the target genes that we identify change at the message level upon restoration of miR-200c. For example, although miR-200c directly targets *ARHGAP19* (Figure S3 in Additional file 1), the message is down-regulated by addition of miR-200c in only 3 of 4 cell lines (Figure S2 in Additional file 1). There are several possible explanations for interference between a miRNA and its mRNA target in some cell lines. The miR-200c target site may be mutated or absent due to a shortening of the 3' UTR [46-49] or there may be RNA binding proteins present in particular cell lines that prevent miR-200c from binding [50]. Importantly, for all of the targets that we follow up on in this study (MSN, FN1 and TrkB), protein levels are affected by miR-200c, indicating that it does have an affect on translation of these genes, regardless of whether it also affects degradation of the message.

Conclusions

In summary, miR-200c inhibits migration and invasion [9-13], stemness [51,52], and chemoresistance [11,12] and we now identify a completely novel role for miR-200c - the ability to reverse anoikis resistance, an important additional step in the metastatic cascade. We identify new targets of miR-200c, which together with previously identified targets, comprise a program of genes normally restricted to cells of mesenchymal or neuronal origin. We specifically pinpoint *MSN* and *FN1* as well as *TrkB* as targets that can respectively mediate the ability of miR-200c to inhibit cell motility and anoikis resistance.

Members of the miR-200 family are down-regulated in breast cancer stem cells and normal mammary gland stem cells [51]. Polycomb complexes facilitate stem cell self-renewal and pluripotency, and both Bmi1, a component of the PRC1 polycomb complex, and Suz12, a component of the PRC2 polycomb complex, have been identified as targets of miR-200 family members [51-53]. It is interesting to speculate as to whether expression of TrkB is involved in the ability of cancer stem cells to resist anoikis.

If feasible, effective *in vivo* delivery of miR-200c could potentially inhibit multiple steps in tumor progression, including tumor formation, cell motility/invasiveness, anoikis resistance and chemoresistance, by virtue of simultaneously repressing multiple, yet specific, targets expressed in carcinoma cells exhibiting an EMT

phenotype. Although one *in vivo* study demonstrated that introduction of miR-200c reduced the ability of primary human breast cancer stem cells to form tumors in immune compromised mice [51], further *in vivo* studies will be necessary to specifically isolate the effects of miR-200 on other steps in the metastatic cascade, such as its potential to reverse anoikis resistance.

Additional material

Additional file 1: Additional experimental data and the sequences of primers used in cloning and qRT-PCR.

Abbreviations

DMEM: Dulbecco's modified eagle's medium; EMT: epithelial to mesenchymal transition; ESR1: estrogen receptor alpha; FAK: focal adhesion kinase; FBS: fetal bovine serum; FN1: fibronectin 1; LEPR: leptin receptor; MSN: moesin; NEAA: non-essential amino acids; poly-HEMA: poly-hydroxyethyl methacrylate; TNBC: triple negative breast cancer; UTR: untranslated region.

Author details

[1]Program in Cancer Biology, Department of Pathology, University of Colorado, Anschutz Medical Campus, Mail Stop 8104, P.O. Box 6511, Aurora, CO, USA. [2]Department of Pathology, University of Colorado, Anschutz Medical Campus, Mail Stop 8104, P.O. Box 6511, Aurora, CO, USA.

Authors' contributions

ENH performed experimental studies. DRC performed array profiling studies and generated the heatmap in Figure 1. All authors contributed intellectual input towards the design, implementation, and interpretation of results. ENH and JRK drafted the manuscript and all authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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The miR-200 and miR-221/222 microRNA Families: Opposing Effects on Epithelial Identity

Erin N. Howe · Dawn R. Cochrane · Jennifer K. Richer

Abstract Carcinogenesis is a complex process during which cells undergo genetic and epigenetic alterations. These changes can lead tumor cells to acquire characteristics that enable movement from the primary site of origin when conditions become unfavorable. Such characteristics include gain of front-rear polarity, increased migration/invasion, and resistance to anoikis, which facilitate tumor survival during metastasis. An epithelial to mesenchymal transition (EMT) constitutes one way that cancer cells can gain traits that promote tumor progression and metastasis. Two microRNA (miRNA) families, the miR-200 and miR-221 families, play crucial opposing roles that affect the differentiation state of breast cancers. These two families are differentially expressed between the luminal A subtype of breast cancer as compared to the less well-differentiated triple negative breast cancers (TNBCs) that exhibit markers indicative of an EMT. The miR-200 family promotes a well-differentiated epithelial phenotype, while high miR-221/222 results in a poorly differentiated, mesenchymal-like phenotype. This review focuses on the mechanisms (specific proven targets) by which these two miRNA families exert opposing effects on cellular plasticity during breast tumorigenesis and metastasis.

Abbreviations

EMT	Epithelial to mesenchymal transition
ZEB1/2	Zinc finger E-box binding homeobox 1/2
UTR	Untranslated Region
MET	Mesenchymal to epithelial transition
MDCK	Madin-Darby Canine Kidney
iPSC	Induced pluripotent stem cell
TGF-β	Transforming growth factor beta
PDGF	Platelet derived growth factor
EGFR	Epidermal growth factor receptor
NCI	National Cancer Institute
VEGF	Vascular endothelial growth factor
ER	Estrogen receptor
MMTV	Murine mammary tumor virus
TRPS1	trichorhinophalangeal 1
PLZF	promyelocytic leukemia zinc finger

E. N. Howe · J. K. Richer
Program in Cancer Biology, University of Colorado,
Anschutz Medical Campus,
Aurora, CO 80045, USA

E. N. Howe · D. R. Cochrane · J. K. Richer (✉)
Department of Pathology, University of Colorado,
Anschutz Medical Campus, 12800 East 19th Avenue,
MS 8104 RC1 N #5122,
Aurora, CO 80045, USA
e-mail: jennifer.richer@ucdenver.edu

Introduction

miRNAs are small (18–25 nucleotide) non-coding RNAs that regulate gene expression post-transcriptionally by binding to the 3′ untranslated region (UTR) of target messenger RNAs (mRNAs) [1], and inhibiting translation or targeting the mRNA for degradation [2]. The extent to which miRNAs regulate the human transcriptome is still under investigation; however, miRNAs can target hundreds of genes, suggesting that their regulatory role may be as significant as that of transcription factors. miRNAs are differentially regulated during development [3–5]. Controlled epithelial to mesenchymal transition (EMT) is a normal process in

development, required for processes such as gastrulation, mammary gland branching, and neural crest formation (reviewed in [6]). However, EMT is a pathological event in cancer that contributes to the gain of aggressive characteristics that facilitate metastasis [7–10]. In cancer EMT, carcinoma cells do not become mesenchymal cells, although there can be a marked loss of epithelial hallmarks and a shift toward mesenchymal and even neuronal gene expression. It is widely believed that acquisition of these characteristics can allow tumor cells to become motile, invasive, and able to intravasate into the blood and lymph vessels and survive the metastatic journey. Transcription factors, such as Twist, Snai1, and ZEB1/2 (Reviewed in [11]) regulate both normal and oncogenic EMT. ZEB1 (zinc finger E-box binding homeobox 1) and ZEB2 (also known as SIP1) directly repress the adherens junction protein E-cadherin [12–14] and other genes involved in polarity and epithelial identity [15, 16].

ZEB1/2 are post-transcriptionally controlled by the miR-200 family of miRNAs [17–19], and ZEB2 is indirectly controlled by the miR-221 family [20]. Indeed, recent studies have identified the miR-200 and miR-221 families as differentially expressed in carcinomas, particularly in breast cancer [20, 21]. Specifically, the miR-200 family is high in the luminal breast cancer subtypes, while miR-221/222 is overexpressed in triple negative breast cancers (TNBCs), particularly those that have undergone EMT. These miRNAs control expression of many genes that define the EMT-like phenotype and likely affect tumor behavior and clinical outcome by influencing metastatic potential. Thus, in this review we focus on the opposing roles of these two miRNA families in controlling differentiation state or epithelial identity in breast cancer.

miR-200 Protection of the Epithelial Phenotype

miR-200 Family Regulation of EMT in Breast Cancer

The miR-200 family of miRNAs is comprised of two polycistronic clusters—miR-200c and miR-141 on chromosome 12 and miR-200b, miR-200a and miR-429 on chromosome 1. miR-200a and miR-141 share a seed sequence, while miR-200b, miR-200c and miR-429 also share a seed sequence, which differs from that of miR-200a/141 by one nucleotide. Because of their sequence similarity, the miRNAs are predicted to share gene targets; however, there is evidence that the two clusters control different regulatory networks even in the same model. In MDA-MB-231 cells the miR-200bc/429 cluster induces G2/M arrest, while miR-200a/141 induces G0/1 arrest [22]. Additionally, miR-200c directly targets and down-regulates the transcription factor ZEB1, while miR-200a does not [23].

The miR-200 family was first discovered to directly target and down-regulate the E-cadherin transcriptional repressors ZEB1 and ZEB2, leading to restoration of an epithelial phenotype in breast cancer cell lines, characterized by an increase in E-cadherin expression, and decreased migration and invasion [17–19]. Expression of the miR-200 family correlates with an epithelial-like phenotype in the National Cancer Institute (NCI) panel of 60 cancer cells lines [19], and suppresses EMT in several additional cancer models, including bladder [24], colorectal [25, 26], and lung [27–30]. Although genes encoding ZEB1/2 are the best-studied targets of the miR-200 family, the small consensus binding sequence of miRNAs results in many bioinformatically predicted targets. The miR-200 family has now been confirmed to directly target other genes involved in various aspects of EMT. One aspect of EMT that has been particularly well studied is the increase in migratory and invasive capacity. Targeting and repression of the genes encoding ZEB1/2 by miR-200c and the resultant increase in E-cadherin decreases migration and invasion; however, direct targeting of genes encoding the actin cytoskeleton associated proteins WAVE3 [31] and MSN [32], and the extracellular matrix component FN1 [32] also contribute to suppression of motility and invasion. The miR-200 family also targets two genes involved in cell cycle control, RND3 [33] and FOG2 [34].

The power of miRNAs lies in their ability to target multiple genes that contribute to a pathway or phenotype. For instance, normal well-differentiated mammary epithelial cells exhibit hallmarks such as E-cadherin and hormone receptor expression, while poorly differentiated breast carcinoma cells loose these characteristics. When carcinoma cells revert towards a less-differentiated state, in addition to losing expression of epithelial hallmarks, they also inappropriately gain expression of proteins that confer the ability to move away from the primary tumor when conditions are harsh (hypoxia, lack of nutrients, and build-up of waste products). The tumor cells must also be able to resist anoikis in order to survive detachment from the basement membrane.

Anoikis resistance is a relatively poorly understood and understudied aspect of EMT. Anoikis is apoptosis induced when cells lose attachment to their native extracellular matrix (ECM), and resistance to anoikis is required for cancer cells to survive as they move away from the primary tumor, and travel through the vasculature or lymphatics to metastatic sites. Data from our lab demonstrate that miR-200c suppresses anoikis resistance through direct targeting of NTRK2, the gene encoding TrkB [32], a receptor tyrosine kinase involved in neuronal development and differentiation. TrkB was first associated with anoikis resistance when it was isolated from a cDNA library screen designed to identify genes capable of conferring anoikis resistance to normal intestinal epithelial cells [35]. TrkB is involved in anoikis resistance in breast cancer [32, 35–38] and is

specifically expressed in TNBCs that have undergone EMT, but not luminal A lines [32].

Resistance to chemotherapy is a critical aspect of tumorigenesis also associated with acquisition of an EMT phenotype. The miR-200 family has been found to be involved in maintaining sensitivity to two classes of chemotherapeutics to date, microtubule targeting agents, and DNA damaging drugs. In aggressive cancer cells resistant to taxanes, restoration of miR-200c increases sensitivity due to its direct targeting of *TUBB3,* the gene encoding class III beta tubulin [39, 40]. TUBB3 is a tubulin isoform aberrantly expressed in several types of carcinomas [41–43], including breast [44, 45], that leads to resistance to taxanes (Reviewed in [46]). Additionally, the miR-200 family is down-regulated in MCF7 cells selected for resistance to cisplatin [47], or doxorubicin [48]. Indeed, miR-200 expression correlates with sensitivity to EGFR blocking agents in bladder cancer, and restoration of miR-200 family members increased sensitivity to EGFR inhibitors in mesenchymal-like cell lines [49]. Additionally, lower expression of miR-200c was observed in a panel of 39 breast cancer patients resistant to chemotherapy [48]. The authors speculate that these effects may be due to the predicted targeting of the multidrug resistance gene 1 by miR-200c, but this remains to be proven. Finally, miR-200c directly targets FAP-1, leading to restoration of sensitivity to CD-95 (Fas)—mediated apoptosis [50]. Thus, the miR-200 family exerts multi-level control over apoptosis in epithelial cells. The family promotes sensitivity to natural apoptotic stimuli, including loss of adhesion and Fas signaling, while also preventing resistance to several classes of therapeutic agents.

While not classically thought of as a characteristic of EMT, an overall decrease in miRNA abundance is found in aggressive cancer cells [51, 52]. Dicer, an enzyme involved in the maturation of miRNAs, is often low in cancers that have undergone EMT [53]. While the mechanism remains to be elucidated, we demonstrated that restoration of miR-200c to TNBC cell lines causes an increase in Dicer protein [21]. Since relatively high levels of Dicer and overall miRNA abundance are characteristic of normal epithelial cells, this is a unique mechanism through which the miR-200 family promotes an epithelial phenotype.

In addition to regulation of EMT, there is emerging evidence that the miR-200 family plays a role in epigenetic regulation and inhibition of stem cell-like qualities in breast, prostate [54, 55], and colorectal cancer cells [26]. Expression of both miR-200 family clusters is down-regulated in stem cells isolated from normal human breast, and murine mammary glands, as well as in stem cells isolated from breast cancer patients [56]. Inhibition of miR-200 leads to an enrichment of the stem cell population, and up-regulation of the miR-200b direct target Suz12, a subunit of the polycomb repressor complex. Increased Suz12 leads to

trimethylation and polycomb-mediated repression of the E-cadherin promoter [57]. Another direct target, the gene encoding class III histone deacetylase, SIRT1, deacetylates histone H3 at the E-cadherin promoter, and miR-200 mediated repression of SIRT further relieves repression of E-cadherin [58]. The miR-200 family also directly targets and represses *Bmi1*, allowing further repression of stemness [26]. Additionally, expression of miR-200c inhibits clonal expansion of stem cells, and prevents tumor formation from patient-derived breast cancer stem cells transplanted into mice [56]. Finally, two important stem cell factors, Sox2 and KLF4 have been found to be down-regulated following restoration of miR-200c [26]. Thus, the miR-200 family controls multiple genes that repress cancer stem cells, leading to restoration of an epithelial phenotype and decreased aggressiveness. The genes and aggressive phenotypes repressed by the miR-200 family are detailed in Fig. 1.

The miR-200 family is highly expressed in luminal A breast cancer cell lines and lost in TNBC lines [21]; however, data from primary and metastatic breast cancer samples are not as clear. Based on the cell line data, it was expected that the miR-200 family would be down-regulated in aggressive tumors and metastases. While this is true in some models, and restoration of miR-200 to a TNBC cell line prevents metastases [59], in other models the miR-200 family positively correlates with metastases [60, 61]. Consistent with the theory that miR-200c positively correlates with a well-differentiated phenotype, the miR-200 family is very low in the poorly differentiated claudin-low subtype of breast cancer, while expression of ZEB1/2, vimentin, and Twist are high and these tumors are enriched for tumor initiating cells, suggesting that the miR-200 family must be down-regulated for formation of an aggressive subpopulation of tumor cells [62]. However, while several profiling studies found that expression of the miR-200 family is lost between normal breast tissue and malignant breast cancers [18, 63] one profiling experiment [64], comparing luminal A, luminal B, basal-like and malignant myoepithelioma, revealed that while the miR-200 family is highly expressed in luminal tumors, it is also highly expressed in basal-like tumors. Only malignant myoepitheliomas showed down-regulation of the miR-200 family, which is consistent with a strong EMT phenotype [64].

Expression of the miR-200 family in metastatic disease has been even more contested. While one group found the miR-200 family to be down-regulated between matched primary versus metastatic breast, colon, lung and bladder cancers [65], another showed that the miR-200 family is over-expressed in matched metastases, and that higher than median expression of several family members correlates with decreased progression free survival in estrogen receptor (ER) positive breast tumors [61]. In contrast, high expression of miR-200b, and low expression of Suz12 can
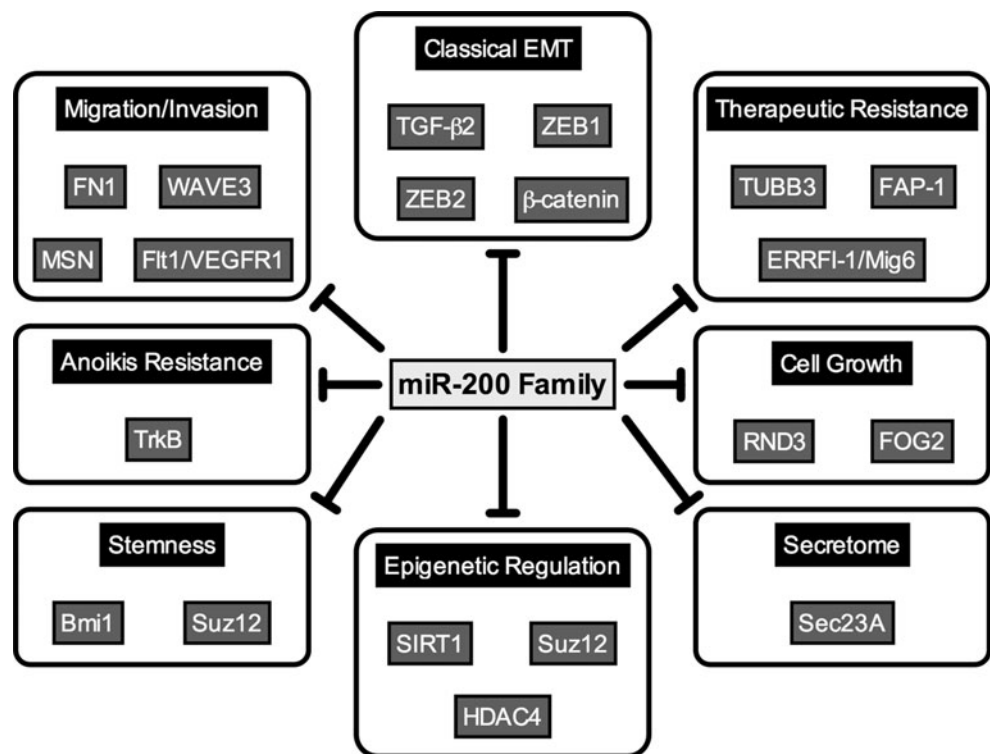
distinguish primary breast tumors from metastases, which express low miR-200b and high Suz12 [57]. Further complicating the matter are two studies performed in syngeneic mouse mammary carcinoma models. In one study, using the 4T1 panel of cell lines, expression of miR-200 in a non-metastatic cell line increased metastasis [60]. Forced expression of miR-200c and miR-141, or all members of the miR-200 family led to increased metastasis in a similar model, the 4TO7 cell line [61]. These studies suggest that expression of the miR-200 family may induce mesenchymal to epithelial transition (MET) during the metastatic cascade. Induction of MET may be necessary for colonization of cells at the metastatic site, which would be consistent with increased expression of the miR-200 family. It is also possible that EMT is not required for metastasis in these models. Another possible explanation is that there are differences in the rate limiting steps of the metastatic cascade across models, which could affect the necessity of MET in colonization. Finally, regulated expression of miR-200 may be important for phenotypic plasticity, and may allow cells to transition between epithelial and mesenchymal states as needed.

miR-200 Family in Plasticity

There is mounting evidence that both EMT and MET are important in the progression of carcinomas, and that carcinoma cells exhibit increased plasticity, allowing them to transition as necessary. Both EMT and MET are required for proper development, and the role of the miR-200 family in transitions between the epithelial and mesenchymal states is becoming clear. During embryonic stem cell differentiation, the miR-200 family is down-regulated by Snai1 and Wnt signaling, and forced expression of miR-200 leads to cells stalling at the epiblast-like stem cell stage of differentiation [66]. The miR-200 family is also regulated by c-Myc in differentiating embryonic stem cells [67].

Forced expression of miR-200c in epithelial cells of the developing mammary gland suppresses ductal growth [56], suggesting that plasticity is required for proper formation of the ducts. Similarly, forced expression of miR-200 in plastic, metastatic lung adenocarcinoma cells reversed plasticity, preventing the cells from undergoing EMT or metastasizing [68]. Manipulation of ZEB1/2 and the miR-200 family in Madin-Darby canine kidney (MDCK) cells leads to EMT and MET, respectively, but the states remain plastic and can be reversed [69]. miRNA profiling of embryonic stem cells, induced pluripotent stem (iPSC) cells, differentiated cells and cancer cells revealed that the pluripotent stem cells formed two clusters, irrespective of the origin of the cells (embryonic versus induced). The miRNAs that distinguished these groups also differentiated normal cells from cancer cells. Expression of miR-92 or miR-200 family members in iPSCs changed their classification status, leading the authors to suggest that the subdivision in pluripotent stem cell states does not reflect their origin, but rather

miRNA and gene expression network [70]. Similarly, the miR-200 family is regulated during reprogramming of somatic cells into iPSCs [71]. Thus, the miR-200 family, as well as EMT-inducing transcription factors, must be expressed in the proper order to allow differentiation of embryonic stem cells.

Regulation of the miR-200 Family

The most potent regulators of the miR-200 family are ZEB1 and ZEB2, which have been demonstrated to target E-boxes in the miR-200 cluster promoters [72, 73]. Another well recognized EMT inducer, transforming growth factor beta (TGF-β), has also been shown to reduce expression of the miR-200 family in transformed human breast epithelial cells [74], murine mammary epithelial cells [75], prostate cancer cells [76], and canine renal MDCK cells, a model of the epithelial phenotype [18, 77]. Indeed, treatment with TGF-β leads to hypermethylation of the miR-200 promoters, potentially through miR-200a-mediated direct targeting of the histone deacetylase SIRT1 [74]. Further study of the role of epigenetic regulation of the family revealed that the promoters are unmethylated in epithelial cells, and in cancer cells that express the family, but heavily methylated in fibroblasts and tumors that do not express the miR-200 family [78, 79]. Furthermore, the permissive epigenetic mark, histone H3 acetylation, is decreased at the miR-200 promoter in cancer cells lacking expression of the family [80], an epigenetic mark potentially influenced by miR-200a direct targeting of HDAC4. Together, this data indicates that while classical EMT-inducers control expression of the miR-200 family in tumorigenesis, epigenetic control is also important, and potentially forms feedback loops through miR-200 control of epigenetic regulators, including SIRT1, HDAC4, and Suz12.

Several other EMT inducers down-regulate the miR-200 family, including platelet derived growth factor (PDGF) [81], long-term treatment with the epidermal growth factor receptor (EGFR) inhibitor gemcitabine [82], and carcinogen induced tumorigenesis [83]. Interestingly, treatment of pancreatic cancer cells with curcumin, or the analog CDF, along with gemcitabine lead to increased miR-200 family expression [81, 84]. Additionally, Akt isoforms leads to differential miRNA expression profiles. Expression of only Akt2 dramatically decreases expression of the miR-200 family, while knockdown of Akt1 induced EMT by reducing expression of the miR-200 family. The authors suggest that the expression of miR-200 family members depends on the ratio of Akt1/Akt2, rather than the overall activity of Akt [85]. To date, the only known activators of miR-200 expression are the tumor suppressors p53 [86, 87], p63, and p73 [88], and ERα [89]. However, there are likely other positive-regulators of the miR-200 family.

miR-221/222 Suppression of the Epithelial Phenotype

miR-221/222 Expression in Breast Cancer and Other Carcinomas

miR-221 and miR-222 are found on the X chromosome and are expressed from a single transcript. For many cancer types, miR-221/222 are considered oncomiRs, and are over-expressed in tumor compared to normal tissue of origin. This expression pattern holds true in breast [63], prostate [90], gastric [91], bladder [92], papillary thyroid carcinoma [93], colorectal cancer [94], melanoma [95], and acute myeloid leukemia [96]. High miR-221/222 expression is associated with increased tumor grade [97, 98] and poor prognosis [99]. High miR-221 is found in prostate cancer cell lines, where it is associated with aggressive phenotypes, such as androgen-independence and neuroendocrine differentiation [90].

Several studies have demonstrated that miR-221/222 directly target ERα [21, 100, 101]. In breast cancer, miR-221/222 negatively correlate with ER status, and are more highly expressed in triple negative cell lines as compared to luminal [20, 21, 100] and the same holds true in clinical samples [21, 102]. Additionally, in the murine mammary tumor virus (MMTV)-c-myc mouse model of mammary carcinoma, miR-222 is increased during tumorigenesis [103]. However, some controversy exists, since one study observed that although miR-221 is overexpressed in TNBCs and is associated with poor disease-free and overall survival, there was no difference in miR-222 expression between breast cancer and normal epithelial tissue [99]. Additionally, another study found that miR-221 expression positively correlated with ER status in breast cancer patient samples, while miR-222 expression did not change between ER positive and ER negative samples [104]. Thus, as with the miR-200 family, although expression of miR-221/222 correlates strongly with specific phenotypes in vitro in breast cancer cell lines, more work is required to fully elucidate the role of the family in human tumors.

miR-221/222 in EMT and Metastasis

Since miR-221/222 are often overexpressed in poorly differentiated, aggressive cancers, it stands to reason that these miRNAs play an active role in promoting EMT. Increasing miR-221 or 222 can affect various characteristics associated with EMT, including increased invasive capacity [90, 105], and anoikis resistance [106]. Low Dicer is characteristic of poorly differentiated cells and cells that have undergone EMT. In TNBC lines, miR-221/222 directly target and repress Dicer1 [21], leading to the possibility that aberrant expression of miR-221/222 leads to decreased Dicer, which in turn leads to a decrease in overall miRNA abundance.

Long term mammosphere culture of MCF7 cells induces EMT, with the resulting cells displaying a basal B phenotype [107]. The cells also exhibit increased expression of stem cell markers (CD44+/CD24-/low), and exhibited stem cell-like characteristics, including chemoresistance. qRT-PCR miRNA profiling demonstrates that miR-200c, -203 and -205 are decreased, while miR-221/222 are increased in the mammosphere cultured cells, with miR-222 increased 20-fold [107]. Thus, although further more exhaustive and rigorous genetic analysis of necessity and sufficiency remains to be performed, it appears that induction of EMT in luminal breast cancer cells involves decreased expression of the miR-200 family and increased expression of miR-221/222. Although miR-221/222 are high in both basal A and B breast cancer, their expression is higher in the basal B subtype, which has a more mesenchymal phenotype [20], consistent with the role of miR-221/222 in EMT. Forced expression of miR-221/222 in luminal breast cancer cells causes a decrease in E-cadherin and an increase in the mesenchymal marker vimentin [20]. Luminal cells expressing miR-221/222 gained a more mesenchymal morphology and had increased migratory and invasive capacity. Conversely, inhibition of miR-221/222 in basal-like cells promoted MET [108]. miR-221/222 promote a mesenchymal phenotype in part by directly targeting trichorhinophalangeal 1 (TRPS1), and keeping its levels low [20]. TRPS1 is a transcriptional repressor that binds to GATA sites that can promote MET [20], and is underexpressed in breast cancers with poor clinical outcome [109]. TRPS1 represses the mesenchymal transcription factor ZEB2 through a GATA site in its promoter. As ZEB2 is a repressor of E-cadherin, this provides a functional link between expression of miR-221/222 and repression of E-cadherin in basal breast cancers [20, 110].

miR-221/222 Control of Proliferation

miR-221/222 positively influence cellular proliferation in many types of cancers. While there are several mechanisms through which increased growth rate is achieved, the best studied is direct targeting of p27[KIP1] [98, 111], and p57[KIP2] [112, 113]. In patient samples, miR-221 or miR-222 levels are often inversely correlated with p27[KIP1] [111, 114–116] or p57[KIP2] [94, 112]. Increasing the expression of miR-221 or miR-222 causes increased proliferation in vitro [111, 114], and increased tumor growth in xenograft tumor models [117]. Conversely, antagonizing miR-221/222 results in decreased proliferation both in vitro [94] and in vivo [118]. In one study, decreased tumor growth was achieved through in vivo administration of cholesterol modified anti-miR-221, which suggests that miR-221 can be a viable therapeutic target for the treatment of aggressive cancers [119].

Direct targets other than p27[KIP1] and p57[KIP2] can also mediate the proliferative effects of miR-221/222. In gastric cancer cells, the proliferative effects of miR-221/222 are partially due to their ability to directly target PTEN [105], and targeting of PTEN is also likely to play an important role in breast carcinomas. Additionally, miR-221/222 directly target ARH1 [120], a tumor suppressor protein decreased in many types of cancers [121–123]. Loss of ARH1 results in increased proliferation, colony formation and invasion [120]. Thus, miR-221/222 promote proliferation by suppressing targets that normally serve to repress proliferative pathways.

miR-221/222 in Resistance to Apoptotic Stimuli

Overexpression of miR-221/222 serves to protect cancer cells against various forms of apoptotic stimuli, including chemotherapeutics, endocrine therapies, radiotherapy and detached growth conditions. MCF7 cells resistant to cisplatin have increased miR-221/222 expression compared to the wild type cells [47]. Antagonizing miR-221 in pancreatic cell lines causes increased apoptosis and sensitized the cells to gemcitabine [124]. miR-221 and miR-222 are increased in taxol resistant cells, and addition of miR-221 to breast cancer cells results in increased survival in response to paclitaxel treatment [125]. One of the mechanisms through which miR-221/222 repress apoptosis is through direct targeting of pro-apototic genes, such as PUMA [126] and BMF [106].

Her2/neu amplified breast cancers tend to be resistant to endocrine therapy [127, 128]. miR-221/222 are high in breast cancers that are positive for Her2/neu, compared to Her2/neu negative breast cancers, and overexpression of miR-221/222 causes MCF7 cells to become tamoxifen resistant [129]. miR-221/222 directly target p27[KIP1] [114] and this is one of the mechanisms through which the cells become tamoxifen-resistant. In xenograft tumors that are resistant to tamoxifen, antagonizing miR-222 sensitizes tumors to tamoxifen [130]. miR-221/222 directly target TIMP3, a tissue metalloproteinase inhibitor that normally inhibits tamoxifen resistant tumor growth. In breast cancer cells that have become resistant to tamoxifen through increased miR-221/222 expression, TIMP3 is repressed, and there is a resultant increase in the expression of metalloproteases ADAM17 and ADAM 10, as well as increased growth factor signaling [130].

While MCF7 cells treated with tamoxifen have slightly decreased levels of miR-221/222, cells treated with fulvestrant, either alone or in combination with E2, have increased miR-221/222 expression [131], likely because ER represses miR-221/222 [101], so degradation of ER after fulvestrant binding could relieve repression of miR-221/222. Inhibition of miR-221/222 activity causes decreased proliferation.

Fulvestrant resistance is explained in part by the downregulation of p27^{KIP1} and p57^{KIP2} [111, 112], and ER [100, 101]. Increased β-catenin contributes to fulvestrant resistance and E2 independent growth [132]. Cells overexpressing miR-221/222 have increased nuclear β-catenin, corresponding to increased β-catenin-mediated transcriptional activity. TGF-β1 blocks proliferation in wild type MCF7s, but not the fulvestrant resistant cells [133, 134]. However, overexpression of miR-221 or miR-222 in wild type cells increases survival in response to TGF-β1, and antagonizing these miRNAs in resistant cells increases sensitivity [131]. Therefore, it is possible that miR-221/222 are involved in switching the effect of TGF-β signaling from tumor suppressive to tumor promotional. The genes and phenotypes regulated by miR-221/222 are depicted in Fig. 2.

Regulation of miR-221/222

There is a negative feedback loop between miR-221/222 and ERα. miR-221/222 directly bind to and downregulate ERα, while ERα binds to estrogen response elements in the promoter of miR-221/222 and represses transcription [101]. Other transcriptional repressors of miR-221/222 function in a cell-type specific manner. For example, in AML cells, the AML1 protein binds to the promoter of miR-221/222 and represses transcription [135]. In melanoma cells, a transcriptional repressor, PLZF (promyelocytic leukemia zinc finger) binds to the promoter of miR-221/222 [136].

FOSL1 (Fra-1) is part of the AP-1 transcription complex and promotes invasiveness and metastatic potential of breast cancers [137–139]. FOSL1 binds an AP-1 site upstream of miR-221/222 and promotes transcription [20]. Activation of the RAS/RAF/MEK pathway increased expression of miR-221/222 in basal breast cancer cells via FOSL1 [20], and activation of the MAPK pathway also increases miR-221/222 expression [D. El-Ashry, Personal Communication].

Interplay Between the miR-200 and miR-221 Families

Perhaps the most convincing evidence that these two families play an important role in epithelial plasticity in breast cancer comes from the White lab, in a study where breast cancer cells were forced to undergo EMT by being grown in mammosphere conditions. The resulting cells had decreased miR-200, and increased miR-221/222 [107]. Collectively, as described above, these two families clearly exert opposing effects on polarity, migration and invasion, proliferation, apoptosis, and differentiation.

ZEB1/2 transcription factors promote a mesenchymal phenotype by repressing genes involved in polarity. Therefore, ZEB1/2 is detrimental to an epithelial phenotype, and it is essential that these genes remain suppressed in differentiated epithelial cells. While they are most definitely repressed at the promoter level, epithelial cells have evolved an additional layer of protection against their expression, which is miR-200 mediated repression at the posttranscriptional level. Conversely, miR-221/222 promote expression of ZEB2 indirectly through TRPS1, and therefore these miRNAs tend to only be expressed in cells that have undergone EMT [20].

miR-221/222 directly target and repress Dicer, while miR-200c increases Dicer by a yet to be identified mechanism [21]. miR-221/222 are more highly expressed in TNBC [21, 100]. miR-103/107 have also been demonstrated to directly target

Fig. 2 Direct targets of miR-221/222. miR-221/222 directly target and down-regulate genes associated with differentiation or tumor suppression. References are included in the text
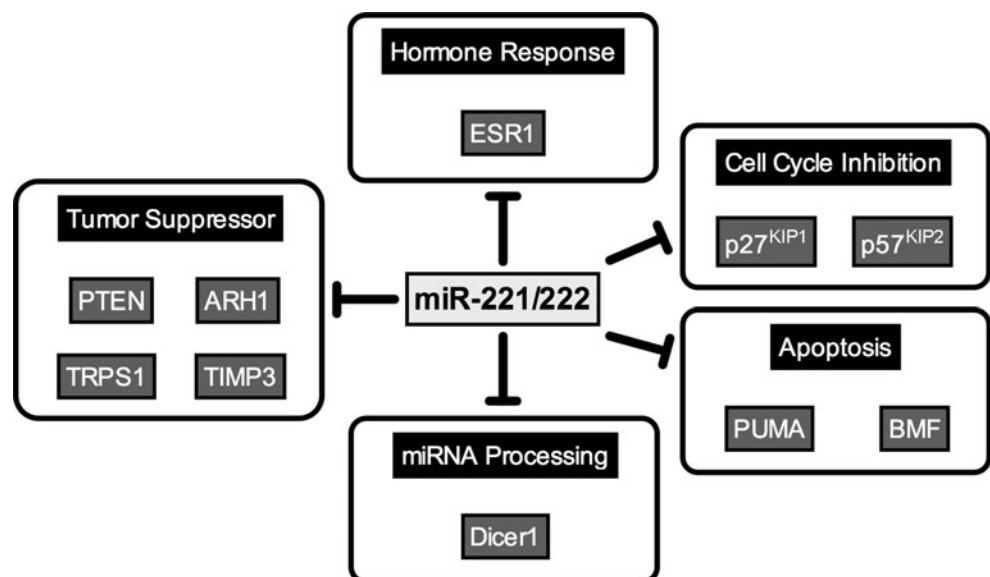
Dicer [140]; however, an inverse correlation between these miRNA and Dicer has not been as well documented as it has for miR-221/222 which are high in tumors in which Dicer levels are low (TNBC). Thus, miR-221/222 may keep Dicer levels low in poorly differentiated breast cancers [21]. Since Dicer is required for the maturation of most miRNAs, this may explain why overall miRNA expression is lower in TNBC than luminal. Dicer is often low in cancers that have undergone EMT [53]. Dicer is clearly lower in TNBC than adjacent normal breast epithelial cells, while in luminal A breast cancers the difference between tumor and normal is much less dramatic (Fig. 3). Interestingly, TAp63 was recently discovered to suppress metastasis by positively regulating Dicer [141]. It is possible that miR-200c increases Dicer through its ability to repress ZEB1, which upregulates deltaNp63 [142], a dominant negative inhibitor of TAp63. Consequently, the miR-221 and miR-200 families may control the global miRNA landscape in normal and cancerous cells by dueling for control of Dicer. Much remains to be explored to fully determine how the influence of these miRNA families over Dicer might control motility and metastasis in normal development and cancer.

Conclusions

The role of miRNAs in tumorigenesis and the power they wield with respect to phenotypic control and tumor behavior is just beginning to be understood. In this review we focus on two of the most dysregulated miRNA families in breast cancer, the miR-200 and miR-221 families. The miR-200 family serves to protect the epithelial phenotype, while simultaneously suppressing EMT and tumorigenesis. The miR-200 family protects against migration/invasion, anoikis and therapeutic resistance, and stem cell-like properties. Conversely, miR-221/222 promote a mesenchymal-like phenotype, and support tumorigenesis. Expression of miR-221/222 inhibits tumor suppressors and genes involved in apoptosis, cell cycle inhibition, and miRNA processing. Both miRNA families impinge on two important pathways: EMT through ZEB1/2, and miRNA processing through Dicer.

These two miRNA families promote dueling phenotypes, thus they are coordinately regulated during cellular transformations such as EMT and MET (Fig. 4). During oncogenic EMT the miR-200 family is strongly down-regulated, while miR-221/222 are highly up-regulated and the reverse is true during MET. This suggests that not only is each miRNA family important for induction of their respective phenotypes, but that the coordinated inverse regulation of these families is required to fully achieve an epithelial or mesenchymal phenotype and associated functional properties. In contrast to their now quite evident role in breast cancer, to date, these miRNA families have not been specifically examined in the normal human breast or mouse mammary gland, although some of their identified targets are clearly relevant in the normal gland.
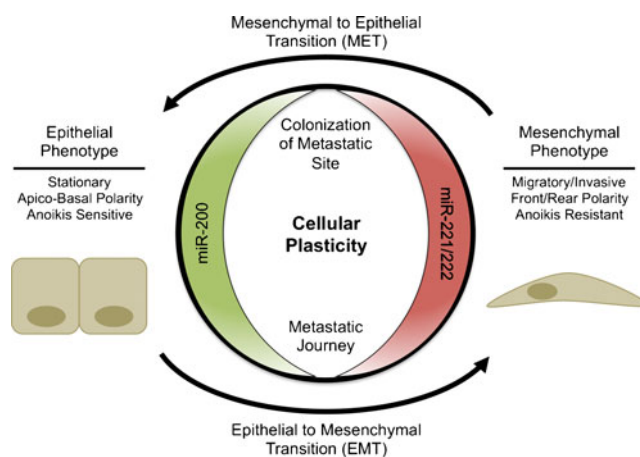

Fig. 3 Dicer protein expression in luminal A and triple negative breast cancer. Formalin-fixed paraffin embedded sections of human breast cancers were stained for Dicer using ab5818 polyclonal antibody (Abcam, Cambridge, MA). Two representative cases each of luminal and triple negative are shown in which adjacent normal glands are present in the same field of vision (top=luminal, bottom=triple negative) with adjacent normal tissue. Red arrows=tumor, black arrows= normal, 200X

Fig. 4 Phenotypic consequences of miR-200 or miR-221/222 expression. In addition to the roles of miR-200 and miR-221/222 in protecting the epithelial or mesenchymal phenotype, respectively, they are also actively regulated during EMT and MET. Green indicates expression of the miRNA is associated with a less aggressive, epithelial phenotype, while red indicates the miRNA is associated with aggressive behavior

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MicroRNAs Link Estrogen Receptor Alpha Status and Dicer Levels in Breast Cancer

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MicroRNAs Link Estrogen Receptor Alpha Status and Dicer Levels in Breast Cancer

**Dawn R. Cochrane · Diana M. Cittelly · Erin N. Howe ·
Nicole S. Spoelstra · Erin L. McKinsey · Kelly LaPara ·
Anthony Elias · Douglas Yee · Jennifer K. Richer**

Abstract To identify microRNAs (miRNAs) associated with
estrogen receptor (ESR1) status, we profiled luminal A,
ESR1+ breast cancer cell lines versus triple negative (TN),
which lack ERα, progesterone receptor and Her2/neu.
Although two thirds of the differentially expressed miRNAs
are higher in ESR1+ breast cancer cells, some miRNAs, such
as miR-222/221 and miR-29a, are dramatically higher in
ESR1− cells (~100- and 16-fold higher, respectively). MiR-
222/221 (which target ESR1 itself) and miR-29a are predicted
to target the 3′ UTR of *Dicer1*. Addition of these miRNAs to
ESR1+ cells reduces Dicer protein, whereas antagonizing
miR-222 in ESR1− cells increases Dicer protein. We
demonstrate via luciferase reporter assays that these miRNAs
directly target the *Dicer1* 3′ UTR. In contrast, miR-200c,
which promotes an epithelial phenotype, is 58-fold higher in
the more well-differentiated ERα+ cells, and restoration of
miR-200c to ERα− cells causes increased Dicer protein,
resulting in increased levels of other mature miRNAs
typically low in ESR1− cells. Together, our findings explain
why Dicer is low in ERα negative breast cancers, since such
cells express high miR-221/222 and miR-29a levels (which
repress Dicer) and low miR-200c (which positively affect
Dicer levels). Furthermore, we find that miR-7, which is
more abundant in ERα+ cells and is estrogen regulated,
targets growth factor receptors and signaling intermediates
such as EGFR, IGF1R, and IRS-2. In summary, miRNAs
differentially expressed in ERα+ versus ERα− breast
cancers actively control some of the most distinguishing
characteristics of the luminal A and TN subtypes, such as
ERα itself, Dicer, and growth factor receptor levels.

Keywords Dicer · miRNA · ESR1 · Epithelial to
mesenchymal transition · Breast cancer

Abbreviations

EMT	Epithelial to mesenchymal transition
ESR1	Estrogen receptor alpha gene
ERα	Estrogen receptor alpha protein
miRNA	MicroRNA
RISC	RNA induced silencing complex
TRBP	Tar RNA-binding protein
TN	Triple negative
UTR	Untranslated region

D. R. Cochrane · D. M. Cittelly · E. N. Howe · N. S. Spoelstra ·
E. L. McKinsey · J. K. Richer (✉)
Department of Pathology, University of Colorado Denver,
Mail Stop 8104, P.O. Box 6511, Aurora, CO 80045, USA
e-mail: Jennifer.Richer@ucdenver.edu

A. Elias
Division of Medical Oncology, Department of Medicine,
University of Colorado Denver,
Aurora, CO 80045, USA

K. LaPara · D. Yee
Department of Medicine, Masonic Cancer Center, University of
Minnesota,
Minneapolis, MN, USA

Introduction

Since specific microRNAs (miRNAs) are capable of regulating
hundreds of mRNAs simultaneously, it was not unexpected to
find that miRNA profiling can distinguish breast cancer
subtypes [5]. MiRNAs function by binding to the 3′
untranslated region (UTR) of their targets and either prevent
translation or cause mRNA degradation. The human RNase
III-type nuclease Dicer performs the final step of biogenesis

of miRNAs in which the pre-miRNA stem loop is cleaved to produce a mature miRNA. The mature miRNA is then incorporated into the RNA induced silencing complex, consisting of Dicer, Tar RNA-binding protein (TRBP), argonaute proteins, and several other proteins, which guide the mature miRNA to specific target mRNAs. Conditional deletion of Dicer enhances transformation and tumorigenesis, and Dicer functions as a haploinsufficient tumor suppressor [36, 37]. Three separate studies of estrogen receptor alpha (ESR1) positive (ESR1+) versus negative (ESR1−) breast cancers found that the majority of differentially expressed miRNAs are less abundant in ESR1− tumors [5, 26, 45]. It is likely that reduced Dicer expression is related to the global down-regulation of the miRNAome observed in cancer, and it is thought that the reduced number and abundance of miRNAs in human cancers reflects an altered differentiation state [7, 43]. Expression of Dicer is lower in breast cancer cell lines and clinical samples that have undergone epithelial to mesenchymal transition (EMT) [23], and Dicer is differentially expressed between ESR1 positive versus negative breast tumors [11]. Similarly, lower Dicer levels are associated with loss of ESR1 in ovarian cancers [18].

Dicer levels are regulated by let-7 via binding sites in the Dicer1 3′ UTR and coding region [19, 60]. MiR-103/107 was recently reported to repress Dicer1 through three sites in the Dicer 3′ UTR [44]. We observed that the 3′ UTR of Dicer1 also contains well-conserved binding sites for miR-221/222, which directly target ESR1 [16, 65] and for miR-29a. We find these to be the most differentially expressed miRNAs higher in ERα- negative versus ERα+ breast cancer cells. We hypothesized that miR-221/222 directly represses not only ESR1, but also Dicer itself, and that miR-29a also directly targets Dicer, possibly explaining why Dicer is lower in ERα negative breast cancers.

In contrast to miR-221/222 and miR-29a, the majority of differentially expressed miRNAs are higher in ERα+ cells, and of these, miR-200c is the most differentially expressed. We previously observed that restoration of miR-200c to dedifferentiated endometrial cancer cells increased Dicer1 mRNA levels [14]. We now demonstrate that restoration of miR-200c to triple negative (TN) breast cancer cells (that lack ERα, progesterone receptors, and Her2neu expression) causes an increase in Dicer protein resulting in an increase in the mature form of some of the miRNAs that are typically lower ERα− cells.

Materials and Methods

Cell Culture and Hormone Treatments

MCF7 and T47D breast cancer cells, which belong to the luminal A subtype, were grown in DMEM, L-glutamine,

penicillin/streptomycin, and fetal bovine serum (FBS). MDA-MB-231 breast cancer cells (triple negative subtype) were grown in MEM containing FBS, HEPES, NEAA, L-glutamine, penicillin/streptomycin, and insulin. BT549 breast cancer cells (triple negative subtype) were grown in RPMI containing FBS and insulin. Hec50 cells were grown in DMEM containing FBS and penicillin/streptomycin. Cells were maintained at 37°C and 5% CO_2. The identity of all cell lines was confirmed using the Identifiler DNA profiling kit (ABI) in the University of Colorado Cancer Center Sequencing Core Facility.

MCF7 cells were grown in phenol red-free media containing charcoal stripped serum for 24 h prior to hormone treatments. The cells were treated with ethanol, 10 nM estradiol, or a combination of 10 nM estradiol and 1 µM ICI 182,780 (ICI, Tocris Bioscience) for 24 h before harvesting total RNA using Trizol (Invitrogen), which retains both small RNA species such as miRNAs and larger RNAs such as mRNAs and rRNAs.

Immunoblotting

Whole cell lysates made with RIPA buffer were separated on SDS PAGE gels and transferred to PVDF membranes, blocked, and probed overnight at 4°C. Primary antibodies used were: ERα (clone AER611,NeoMarkers), E-cadherin (clone NCH-38, DAKO), ZEB1 (rabbit polyclonal, Dr. Doug Darling, University of Louisville); N-cadherin (clone 13A9, Upstate), Vimentin (clone V9, Sigma), Dicer (rabbit polyclonal, Sigma), α-tubulin (clone B-5-1-2, Sigma), EGFR (rabbit polyclonal, Cell Signaling Technology), IGF1Rβ (rabbit polyclonal (C-20), Santa Cruz Biotechnology), IRS-1 [56], IRS-2 (rabbit polyclonal (H-205), Santa Cruz Biotechnology), ERK1/2 (MAPK), phospho-specific and total (rabbit polyclonal, Cell Signaling Technology). After incubation with HRP-conjugated secondary antibodies, results were detected using Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer).

MiRNA Microarray Profiling

Total RNA was prepared using Trizol (Invitrogen). Labeling, hybridization to miRNA microarray slides, and feature extraction was performed by ThermoFisher using the Agilent miRNA microarray platform containing all miRNAs in the Sanger version 10 database. Each miRNA probe is spotted in seven locations to allow for statistical analysis to be performed. Relative intensity data for the multiple probes for each miRNA was subjected to statistical filtering. Probes with p values ≤0.05 in at least two of the eight slides were retained for further analysis. For the luminal versus triple negative screen, the filtered array data was analyzed and clustering was performed using GeneSpring GX 10 (Agilent

Technologies). Data was filtered using a twofold change cutoff and a *p* value of 0.05 (ANOVA, Benjamini Hochberg FDR multiple testing correction). For the graphical representation of the data, averages were taken for T47D and MCF7 to generate the ERα+ values and averages for MDA-MB-231 and BT549 were used to generate the ERα− values.

Real Time RT-PCR

TaqMan MiRNA Reverse Transcription kit was used to generate cDNA from total RNA using a miR-7, miR-29a, miR-221, miR-22, miR-193, miR-148a, or U6 specific primers (Applied Biosystems). For normalization, real time RT-PCR was performed on the cDNA using 18S rRNA primers and probe (Applied Biosystems). For miR-34a, miR-19b, miR-20a, and miR-106a, poly A tailing and reverse transcription was performed using the NCode miRNA qRT-PCR kit (Invitrogen). SYBR green real time RT-PCR was performed using the Universal Forward Primer (Invitrogen) and a miRNA specific primer. For normalization, levels of β-actin were quantified using gene-specific primers. The relative miRNA levels were calculated using the comparative Ct method ($\Delta\Delta$Ct). Briefly, the Ct (cycle threshold) values for the rRNA, U6, or actin were subtracted from Ct values of the miRNA to achieve the ΔCt value. The $2^{-\Delta Ct}$ was calculated and then divided by a control sample to achieve the relative miRNA levels ($\Delta\Delta$Ct). Reported values are the means and standard errors of three biological replicates.

Immunohistochemistry

Sections were cut at 4 μm and heat immobilized. After deparaffinization and antigen retrieval, endogenous peroxidase was blocked. Sections were incubated with primary antibody for 1 h. Primary antibody used was Estrogen Receptor alpha (clone 1D5, Dako). Vectastain Elite ABC kit (Vector Labs) was used for serum blocking and antibody detection, followed by incubation with 3, 3′-diaminobenzidine (Dako) for protein visualization.

In Situ Hybridization

Sections of paraffin-embedded specimens were deparaffinized in xylene, rehydrated with ethanol, and subjected to proteinase K digestion (10 μg/ml, 5 min) and 0.2% glycine treatment. Samples were refixed in 4% paraformaldehyde and treated with acetylation solution, rinsing with PBS between treatments. Slides were prehybridized at 53°C for 1 h in hybridization solution (50% formamide, 5× SSC, 0.5 mg/ml yeast tRNA, heparin). Double-DIG LNA-modified DNA probe complementary to mature miR-222

or scramble control (Exiqon) at 40 nM in hybridization solution was incubated overnight at 50°C and washed in SSC at increasing stringency (5 to 0.2× SCC) at 50°C, then with PBST at room temperature. Slides were incubated for 1 h with blocking solution (TBST, 1% BSA, 0.1% FBS) and then 1 h with 1:2,000 dilution of anti-digoxigenin antibody (Roche). After washing with TBST, slides were incubated with AP solution for 15 min and then BM purple-substrate until color development was evident (24 to 72 h). The reaction was stopped when positive controls developed purple color while negative controls remained colorless.

Transfections

Transfections of 50 nM miR-221, miR-222, miR-29a, miR-200c, and miR-7 mimics (Ambion) were performed as described previously [14]. Protein and RNA were harvested 72 h post-transfection.

Luciferase Assays

Fragments of the ESR1 3′ UTR containing the putative binding sites for miR-203, miR-221, and miR-22 (nucleotides 2126–2472, ESR1 A) and a region that does not contain any miR-221 or miR-22 binding sites (nucleotides 3585–4249, ESR1 B) were amplified by PCR from HeLa genomic DNA (New England Biolabs). Fragments of the *Dicer1* 3′ UTR containing putative binding sites for miR-29a (nucleotides 1096–1752, Dicer 3′ UTR A) or miR-221/222 (nucleotides 2636–3028, Dicer 3′ UTR B) were also amplified. These fragments were cloned into pMIR-REPORT (Ambion). Site-directed mutagenesis was used to introduce a three-nucleotide mutation into the location where the miRNA see sequence binds (Dicer A mut and Dicer B mut). For ESR1, MCF7s were used, and for Dicer, Hec50 (an endometrial cell line) was used. Cells (20,000) per well were plated into a 96 well plate. The cells were mock transfected, transfected with 50 nM negative control mimic, with mimics for miR-221 or miR-222 (for the ESR1 fragments), miR-29a, miR-222, or antagonists for miR-29a or miR-222 (for the Dicer fragments) (Dharmacon). After 24 h, firefly reporter plasmid (0.196 μg) and a *Renilla* luciferase normalization plasmid pRL-SV40 (0.004 μg) were introduced using Lipofectamine 2000. Cells were harvested 48 h later for analysis using the Dual Luciferase Reporter assay system (Promega).

Generation of Stable Cell Lines

Cell lines stably expressing shRNAs targeting ZEB1 or luciferase were generated using SMARTvectorTM shRNA Lentiviral Particles (Thermo Scientific Dharmacon) as described previously [15]. For stable expression of the miR-222

Fig. 1 MiRNAs differentially expressed in luminal versus triple negative breast cancer cell lines. **a** Protein expression of epithelial markers (ERα, E-cadherin) and mesenchymal markers (ZEB1, vimentin, N-cadherin) in luminal A (MCF7 and T47D) and triple negative (MDA-MB-231 and BT549) cell lines. PSTAIR is shown as a loading control. **b** Immunoblot of Dicer in luminal A (MCF7 and T47D) and triple negative (MDA-MB-231 and BT549) cells, with α-tubulin as a loading control. **c** MiRNA microarray analysis performed in luminal A versus triple negative cell lines. Biological duplicate samples for each cell line were hybridized to Agilent miRNA microarrays. Heatmap of miRNAs that exhibit a 1.5-fold differential expression between luminal and triple negative cell lines
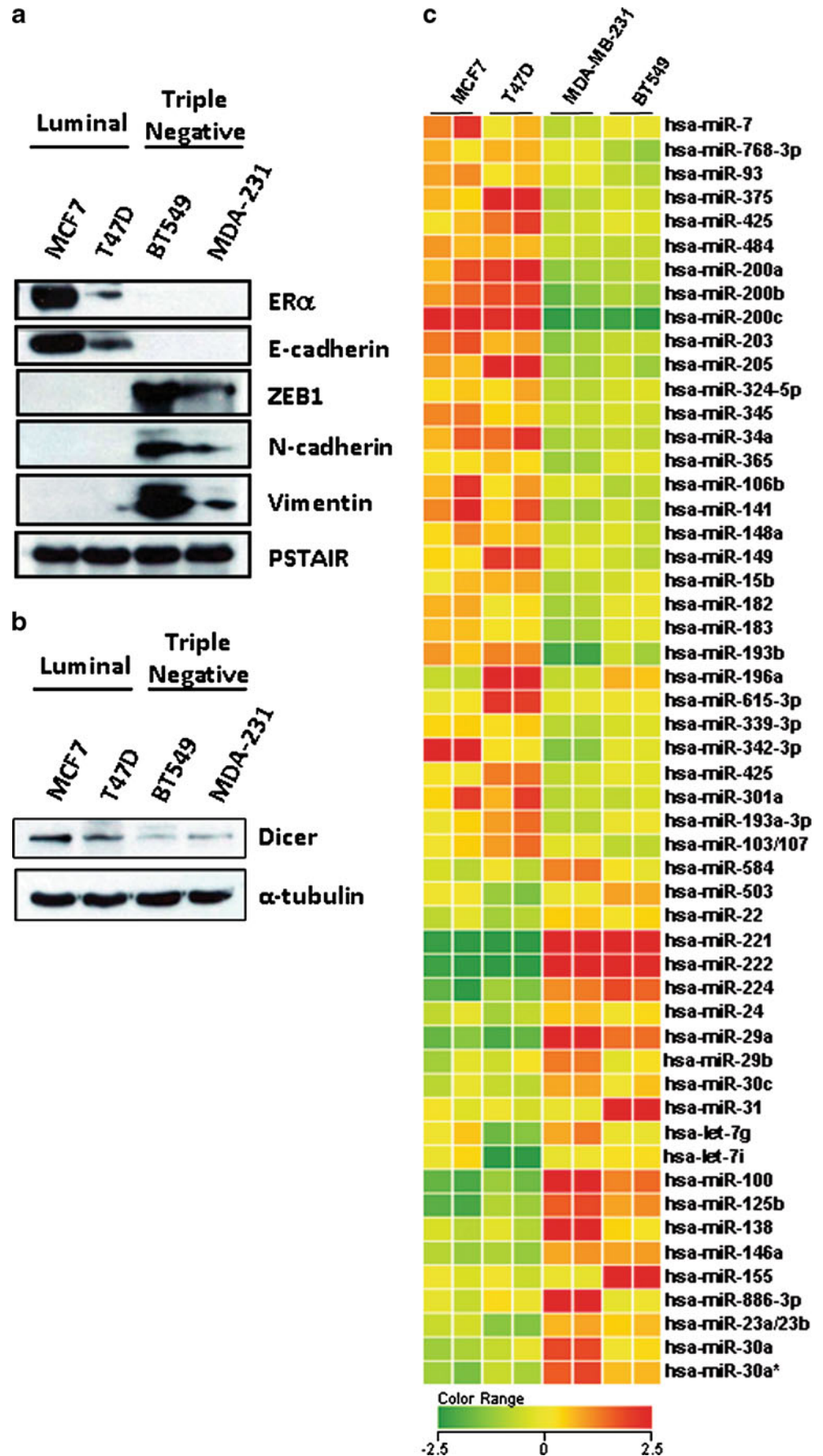
antagonist, pmiR-222-Zip, or pGreenPuro Scramble Control (System Biosciences Inc.), lentiviral vectors were packaged in 293FT cells and virus was harvested after 48 h. Virus was added to MDA-MB-231 or BT549 cells at 1:10 or 1:1 virus: media and selection was performed using puromycin.

Results

MiRNAs are Differentially Expressed in ERα+ and ERα− Breast Cancer Cell Lines, the Majority Being More Abundant in ERα+ Cells

We performed miRNA microarray profiling of two breast cancer cell lines representing the luminal A subtype (MCF7 and T47D) and two representing the TN subtype (MDA-MB-231 and BT549). Luminal A cells are relatively well differentiated and retain expression of ERα and E-cadherin, while the TN cells, in particular the basal-like or claudin

low subset (which the MDA-MB-231 and BT549 cells represent) [24], have lost expression of these luminal markers and express mesenchymal markers such as ZEB1, N-cadherin, and vimentin (Fig. 1a) and have thus undergone EMT. We also observe that Dicer levels are higher in luminal A cell lines (MCF7 and T47D) compared to TN cell lines (MDA-231 and BT549) (Fig. 1b). Previous reports have indicated that *Dicer1* mRNA expression is lower in carcinoma cells with a mesenchymal phenotype [11, 23]. To determine if this is true in large scale datasets, we mined four breast cancer microarray datasets for *Dicer1* expression separating the data into ESR1+ and ESR1− cohorts. *Dicer1* mRNA levels are significantly lower in the ESR1− breast cancers in all four studies (Supplemental Fig. 1). We find that 53 miRNAs are differentially expressed in luminal A versus TN cell lines (Fig. 1c). Consistent with previous reports that the majority of miRNAs are downregulated in aggressive breast cancers [5, 26, 45], two thirds (31) of the 53 differentially expressed
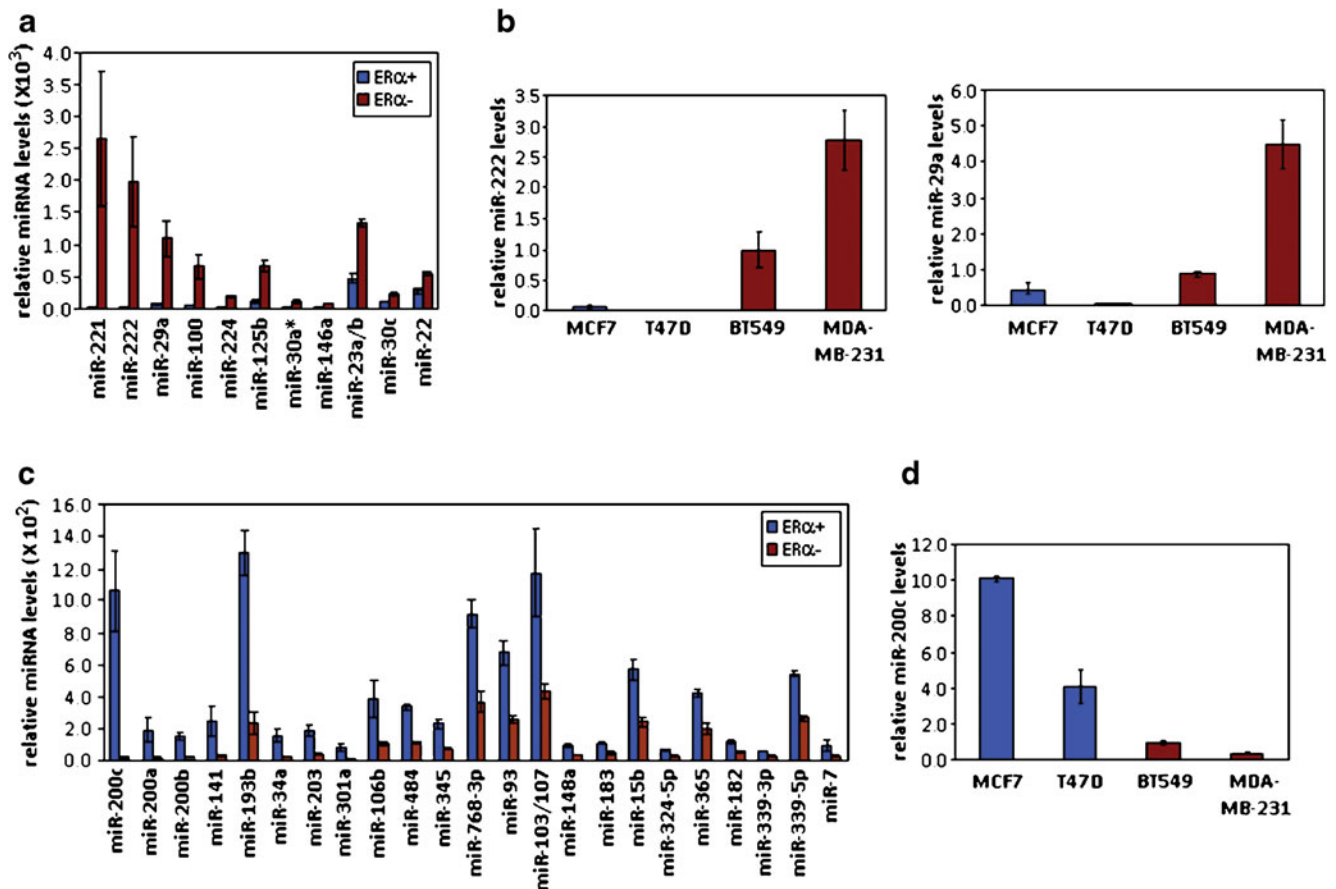

Fig. 2 MiR-222 and miR-29a are higher in ERα- cell lines compared to ERα+. **a** Graphical representation of miRNAs more highly expressed in ERα+ breast cancer cell lines that are 1.5 different with *P*<0.05. The *blue bars* are the average values for the ERα+ cells (T47D and MCF7), while the *red bars* are the average values for the ERα− cells (MDA-MB-231 and BT549). **b** Real time PCR validation of miR-222 (*left*) and miR-29a (*right*) expression levels, relative to the BT549 values. *Error bars* represent standard error of the mean. **c** Graphical representation of miRNAs more highly expressed in ERα+ cell lines and (**d**) real time PCR validation of miR-200c expression levels, relative to BT549 values. *Error bars* represent standard error of the mean

miRNAs that we identify are higher in ERα+ cells compared to ERα− (Fig. 1c).

MiR-221/222 and miR-29a are the Most Differentially Expressed miRNAs More Abundant in ERα− Cells

Of the miRNAs higher in ERα− cells, the most differentially expressed and most abundant were the highly homologous miR-221 and miR-222, as well as miR-29a (Fig. 2a). Real time PCR on independent samples confirmed that these miRNAs are more abundant in ERα− (Fig. 2b). The most differentially expressed miRNA that is higher in ERα+ cells is miR-200c, which has been previously demonstrated to be lost in high grade cancers [6, 14, 58], followed by the other miR-200 family members (Fig. 2c). The differential expression of miR-200c in ERα+ and ERα− was also confirmed by RT-PCR (Fig. 2d).

MiR-222 and miR-22 Act Additively to Decrease ESR1

Study of the miRNAs predicted to target the *ESR1* 3′ UTR using Miranda, PicTar, and Targetscan target prediction programs indicates that the miR-221/222 and miR-22 target sites are in close proximity (Fig. 3a). These miRNAs have been previously reported to target *ESR1* [48, 52, 65], and we find that they are both higher in ESR1− cell lines (indicated in red). In contrast, we find that many of the other miRNAs predicted to bind the ESR1 3′ UTR are paradoxically more abundant in ESR1+ (indicated in blue) (Fig. 3a). MiRNAs can cooperate to downregulate a target when their binding sites are closely located as the miR-221/222 and miR-22 sites are [10, 57]. While the addition of each miRNA alone to ESR1+ MCF7 cells causes a marked decrease in ERα protein, an additive effect was observed when both miRNAs are combined (Fig. 3b). To demonstrate direct targeting of the miRNAs to the 3′ UTR of the *ESR1* transcript, we utilized luciferase reporter assays in which two regions of the *ESR1* 3′ UTR (termed ESR1 A and B) were cloned into the region 3′ of the luciferase gene on a reporter vector. The region denoted ESR1 A contains the miR-221/222 and miR-22 target sites, while ESR1 B is predicted not to be targeted by miR-221/222, miR-22, or any of the miRNA that are higher in ERα− cells and serves as a negative control. Empty luciferase reporter vector containing no target sequences downstream of luciferase also serves as a negative control. These vectors were transfected into MCF7 cells (which lack miR-221/222) in combination with either a scrambled negative control, the miR-22 or miR-221 mimics alone or both miR-22 and miR-221 in combination (Fig. 3c). We observe an 18.8% decrease in luciferase activity in the cells transfected with ESR1 A and the miR-22 mimic compared to the scrambled negative control. With the miR-221 mimic, there is a 13.7%

decrease in luciferase activity versus the negative control. When both mimics are combined, we observe a 32.7% decrease in luciferase activity, demonstrating an additive effect when the two miRNAs are combined. We performed in situ hybridization for miR-222 (the homolog of miR-221) on luminal A versus TN (confirmed ERα, progesterone receptor and Her2/neu negative) breast cancer clinical samples obtained from the University of Colorado Breast Cancer Tissue Bank (protocol 04-0066). Figure 3c shows in situ hybridization for miR-222 and IHC for ERα on five representative TN and five luminal A breast cancers. We find miR-222 expression only in TN tumors, whereas in luminal A tumors, miR-222 staining is absent (Fig. 3c). Examples of the levels of staining in cells positive (MDA-MB-231) and negative (MCF7) for miR-222 are shown in Supplemental Fig. 2.

MiR-221, -222, and miR-29a Target *Dicer1*

In order to test our hypothesis that a direct link exists between miRNAs overexpressed in ESR1− cells and low Dicer levels, we transfected mimics for miR-221/222 and miR-29a into ESR1+ T47D cells and found that they each decrease Dicer protein to almost undetectable levels (Fig. 4a). Real time PCR for each of these miRNAs in the transfected cells is shown in Supplemental Fig. 3. Furthermore, inhibition of miR-222 by stable expression of the antagonist miR-222-ZIP results in increased Dicer protein in both MDA-MB-231 and BT549 cells (Fig. 4b). The *Dicer1* 3′ UTR contains well-conserved predicted target sites for miR-221/222 and miR-29a in close proximity to

Fig. 3 MiR-22 and miR-221 act additively to decrease ERα levels. **a** Map of 3′ UTR of *ESR1* showing putative miRNA binding sites. Target sites for miRNAs that have higher expression in ESR1− cells are circled in *red*, while target sites for miRNAs more highly expressed in ESR1+ cells are in *blue*. **b** Western blot of MCF7 cells treated with a mock transfection, a scrambled negative control, miR-22 mimic, a miR-221 mimic or a combination of miR-221 and miR-22 mimics. Protein was harvested 72 h after transfection, transferred, and probed for ERα and α-tubulin as a loading control. The experiment was repeated three times; shown is a representative blot. **c** The region of the *ESR1* 3′ UTR containing the miR-22 and 221 binding sites (ESR1 A) and a separate region of the *ESR1* 3′ UTR not containing miR-22 or miR-221 binding sites (ESR1 B) were each cloned downstream of luciferase in a reporter vector. These constructs or the empty reporter vector were transfected into cells treated with a scrambled negative control, miR-22 mimic, miR-221 mimic or both, and a luciferase assay performed. *Error bars* represent standard error of the mean for five replicates. *Single asterisk* indicates a statistically significant difference, $P<0.05$, compared to ESR1 A and *two asterisks* indicate a statistically significant difference, $P<0.01$, compared to EV and ESR1 B (two-way ANOVA, Bonferroni post test). **d** In situ hybridization for miR-222 and immunohistochemistry for ESR1 in luminal and triple negative clinical samples (ESR1 staining is *brown* and miR-222 staining is *purple*). MiR-222 in situ staining with a scrambled negative control is shown at the *bottom* (×400 magnification)
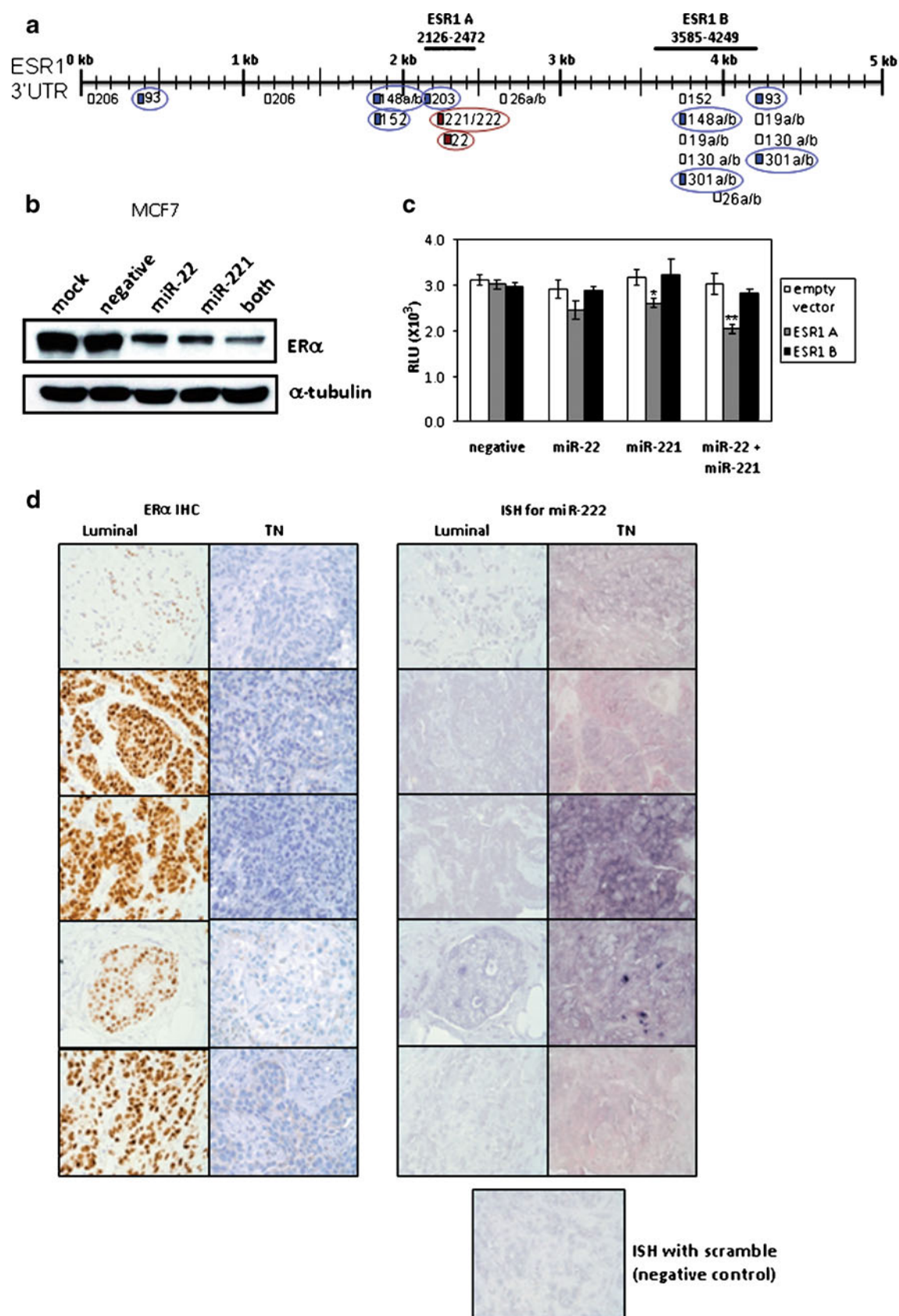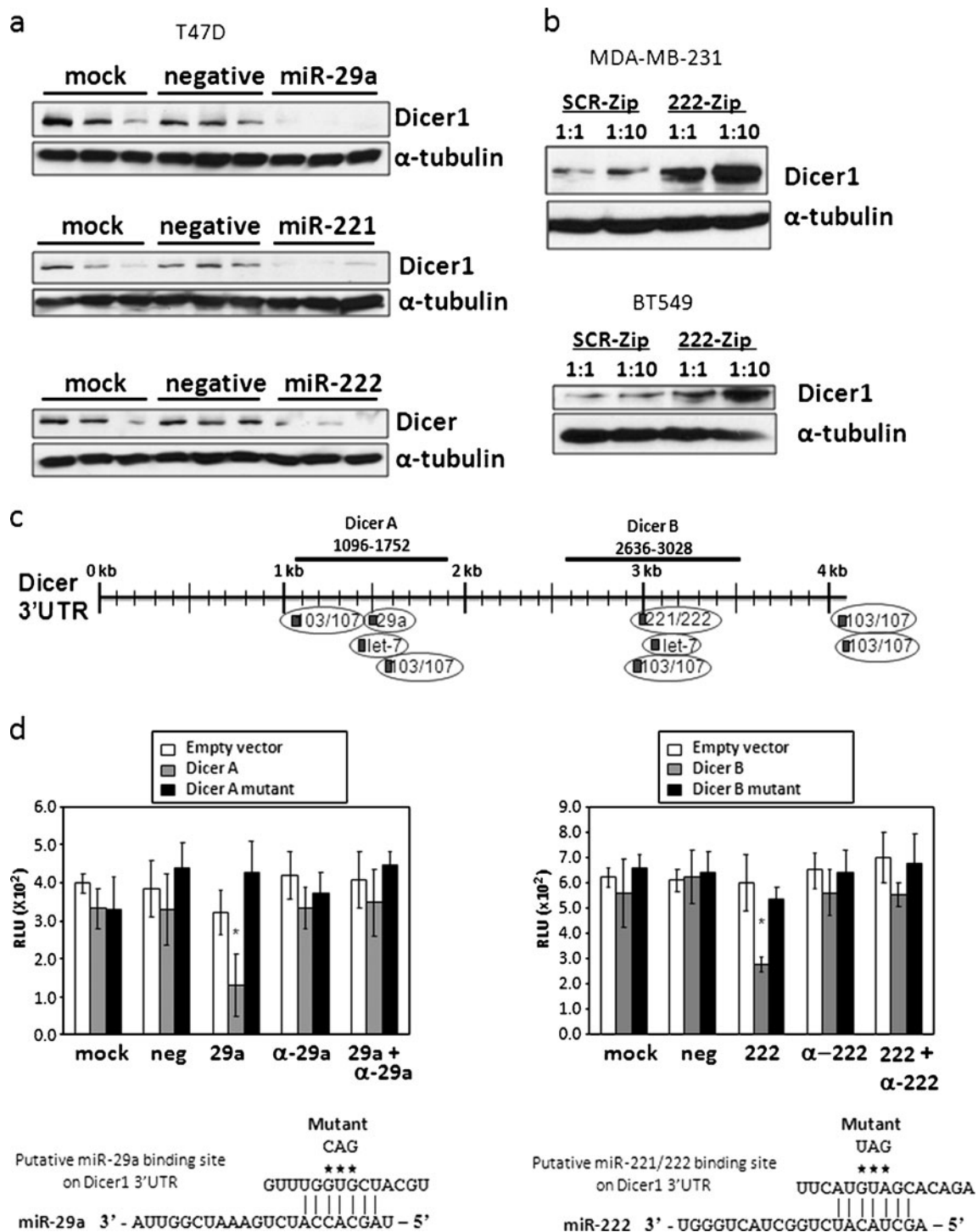
Fig. 4 MiR-29a and miR-221 or 222 reduce Dicer protein expression by directly targeting Dicer. **a** Immunoblot for Dicer in T47D cells mock transfected, transfected with a scrambled negative control, miR-29a, miR-221, or miR-222 mimic. **b** Immunoblot for Dicer in MDA-MB-231 and BT529 cells stably expressing miR-222 antagonist (222-Zip) or scrambled negative control (SCR-Zip). **c** Map of 3′ UTR of *Dicer1* showing putative miRNA binding sites. Target sites for miRNAs that have higher expression in triple negative cells are in *red*, target sites for miRNAs more highly expressed in luminal cells are in *blue*. **d** Luciferase assay on fragments of the *Dicer1* 3′ UTR containing the miR-29a binding site (*left*) or the miR-221/222 binding site (*right*), fragments containing mutated binding sites or an empty vector. Hec50 cells were mock transfected, transfected with a scrambled negative control, miR-29a or miR-222 mimics, antagonists of miR-29a or miR-222, or a combination of both. The mutations introduced into the putative miRNA binding sites are pictured *below* the graphs. *Asterisk* indicates $P<0.05$, Student's t test

previously characterized let-7 sites (Fig. 4c). We cloned two regions of the *Dicer1* 3′ UTR containing the putative miR-29a or miR-221/222 binding sites (Dicer A and B) as well as those same fragments containing mutated miRNA target sites (Dicer A and B mut) downstream of luciferase (Fig. 4c). There is a decrease in luciferase activity only in the cells treated with miR-29a or miR-222 with the appropriate Dicer construct (Fig. 4d). This effect is abrogated when the target site is mutated, showing that the binding site is functional. Furthermore, antagonists of miR-29a and miR-222 are able to prevent binding, showing that the effect is specific to these miRNAs.

Dicer is Positively Regulated by miR-200c

We previously observed that miR-200c increases *Dicer1* message [14]. We also observed that due to reciprocal repression between miR-200c and ZEB1 [6], reducing ZEB1 expression with shRNA causes an increase in endogenous miR-200c [15]. We find that increasing endogenous miR-200c in MDA-MB-231 cells by using shZEB (which we have shown previously to relieve repression of endogenous miR-200c [15] (Supplemental Fig. 4) or adding exogenous miR-200c mimic increases Dicer protein in MDA-MB-231 and BT549 cells (Fig. 5a, b). Since miRNAs usually function in a repressive manner, the mechanism by which miR-200c increases Dicer protein is likely through an indirect mechanism. We hypothesized that since many mature miRNAs are low in ERα− cells

(perhaps due to inefficient maturation as a result of low Dicer), increasing Dicer might increase levels of mature miRNAs typically low in the TN cells. To test this hypothesis we measured levels of the mature forms of miRNAs originally observed to be low in TN cells in cells transfected with miR-200c mimic (in which endogenous Dicer levels had increased). We find that in MDA-MB-231 cells, miR-193b, miR-34a, and miR-148a are increased with miR-200c mimic compared to the negative control (Fig. 5a). Several other miRNAs (miR-15b, miR-103, miR-301a, and miR-106b), which we also find to be more abundant in ERα+ cells, also demonstrated increased levels in the miR-200c treated cells (data not shown). In BT549 cells, we observe an increase in miR-34a, miR-148a, and miR-301a when transfected with the miR-200c mimic (Fig. 5b). However, addition of miR-200c does not repress miR-221/222 levels (data not shown).

MiR-7 is an Estrogen-Regulated miRNA that Targets Growth Factor Receptors Overexpressed in TN Breast Cancers

To identify miRNAs not only associated with ERα positivity, but actually regulated by estradiol-bound ERα, we performed miRNA microarray profiling of MCF7 cells treated for 24 h with 10 nM estradiol or ethanol vehicle control (Fig. 6a). At 24 h, the expression of six miRNAs significantly decreased while eight significantly increased with estrogen treatment. MiR-7 and miR-324-5p are both
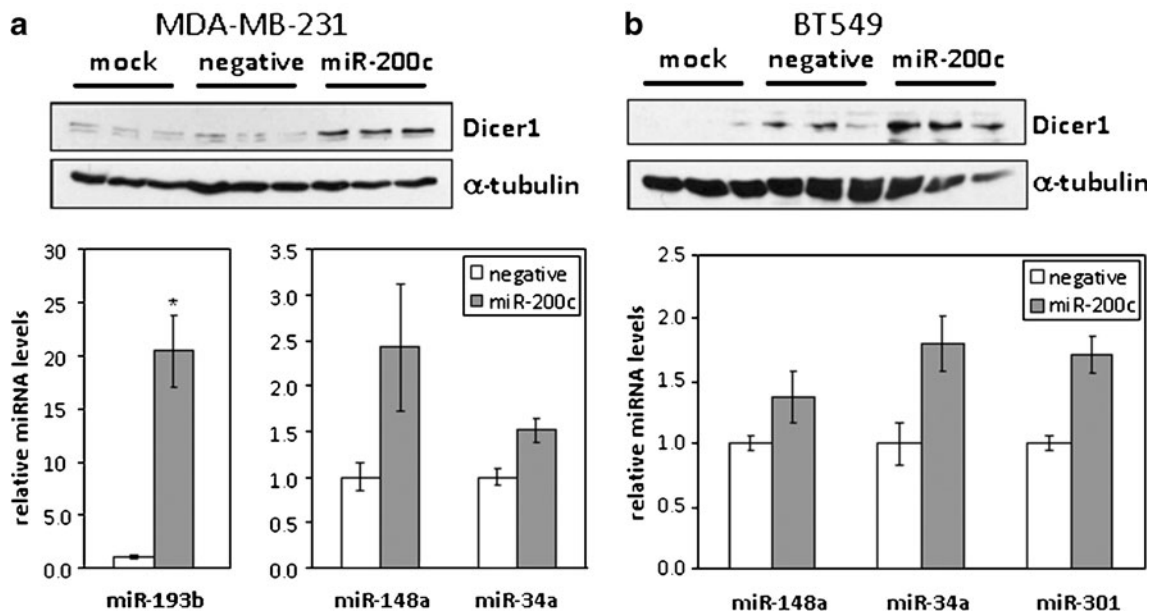

Fig. 5 Restoration of miR-200c to ESR1− breast cancer cells increases Dicer protein. Immunoblot for Dicer in MDA-MB-231 (**a**) and BT549 (**b**) cells mock transfected, transfected with a scrambled negative control or a miR-200c mimic for 72 h. Bottom, real time PCR for miR-193b, miR-34a, and miR-148a in MDA-MB-231 cells and mR-34a, miR-148a, and miR-301a in BT549 cells transfected with a scrambled negative control or a miR-200c mimic. An *asterisk* indicates $P<0.05$, Student's *t* test
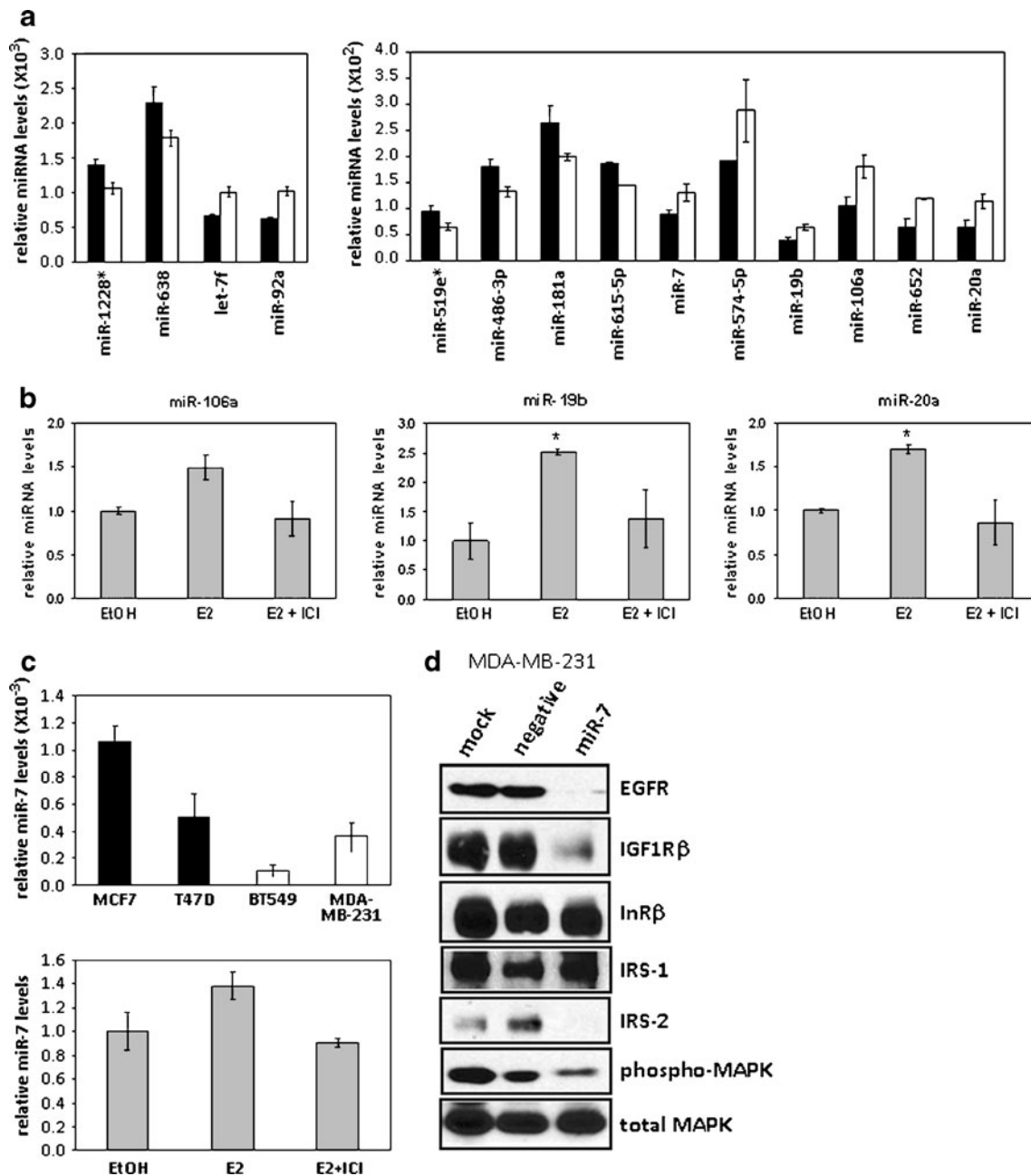
Fig. 6 MiR-7, which is associated with ERα positivity and is upregulated by estrogen, targets growth factor receptors and downstream signaling molecules. **a** MiRNA microarray analysis of miRNAs differentially regulated by 10 nM estradiol (*white bars*) at 24 h versus the ethanol vehicle controls (*black bars*) in MCF7 cells. Shown are the miRNAs that have a 1.5-fold difference and $P<0.05$. *Error bars* represent the range of biological duplicates. **b** Real time PCR for miR-106a, miR-19b, and miR-20a in MCF7 cells treated with the ethanol vehicle control, 10 nM estradiol (E2) or estradiol, and 1 μM ICI (E2+ICI) for 24 h. Shown are the averages of three replicate samples, and *error bars* represent standard error of the mean. *Asterisk* indicates a statistically significant difference between E2 treated and the vehicle control, with $P<0.05$, Student's *t* test. **c** Real time PCR for miR-7 was performed in two ESR1+ cell lines (MCF7 and T47D) and two ESR1− cell lines (BT549 and MDA-MB-231), *top*, and in cells treated with the ethanol vehicle control, 10 nM estradiol (E2) or estradiol, and 1 μM ICI (E2+ICI) for 24 h, *bottom*. Shown are the averages of three replicate samples, and *error bars* represent standard error of the mean. **d** MDA-MB-231 cells were mock transfected, transfected with a scrambled negative control, or a miR-7 mimic for 72 h. Protein was harvested and blots probed for EGFR, IGF1Rβ, InRβ, IRS-1, IRS-2, phospho-MAPK ,and total MAPK (also used as a loading control)

higher in ESR1+ cells and positively regulated by estrogen. We confirmed in independent samples by real time RT-PCR that several members of the miR-17-92 cluster or the paralog miR-106a-363 cluster are estrogen regulated (Fig. 6b). MiR-20a and one of the copies of miR-19b appear in the miR-17-92 cluster, while miR-106a and the other copy of miR-19b are in the miR-106a-363 cluster. We confirm that miR-7 is expressed more highly in ESR1+ cell lines and estrogen increases miR-7 levels in an ESR1-dependent manner (Fig. 6c). Bioinformatic analysis predicts epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF1R), and insulin receptor substrates 1 and 2 (IRS-1, IRS-2) as putative miR-7 targets. IGF1R contains three predicted miR-7 binding sites and IRS-2 contains two putative binding sites. Addition of a miR-7 mimic to ESR1− cells dramatically decreased EGFR and IGF1Rβ at the protein level with no effect on the insulin receptor (Fig. 6d). There is also a profound decrease in IRS-2 protein following the addition of the miR-7 mimic, but no effect on IRS-1. Finally, we observe a decrease in the amount of phosphorylated ERK1/2 (MAPK) with no effect on total MAPK.

A schematic of the regulation of key distinguishing features of TN versus luminal cancers by miRNAs is shown in Fig. 7. Both Dicer and ERα are expressed at high levels in luminal breast cancers and are markers of a differentiated epithelial phenotype. MiR-221 and miR-222 are high in TN breast cancers and target both Dicer and ERα. MiR-29a is also high in TN breast cancers and targets Dicer. MiR-200c is high in luminal breast cancers and increases Dicer expression. MiR-7 is expressed at high levels in luminal A cells and limits the expression of growth factors receptors

such as EGFR and IGF1R that are often overexpressed in TN cancers, and the signaling intermediate IRS-2.

Discussion

MiRNA profiling of ERα+ versus ERα− breast cancer cell lines reveals that the majority of miRNAs are lower in the ERα− cells. This is consistent with previous reports of a global decrease in miRNA expression in cancer [5, 26, 45]. While many miRNAs are located in fragile sites that are often lost in cancer [7], it is also possible that decreased expression of component(s) of the miRNA processing machinery prevent efficient miRNA processing. Indeed, cancer cells can have decreased expression of mature miRNAs, while maintaining expression of precursors [39, 47, 59] and impairment of miRNA processing results in transformation and increased tumorigenesis [36, 37]. MiRNAs are essential for differentiation and maintenance of a differentiated state. Dicer-deficient stem cells are unable to properly differentiate [27, 28], and loss of Dicer causes apoptosis in differentiated neural crest cells [63] and prostate epithelial cells [64]. The loss of differentiation and increased aggressive behavior that accompanies EMT may be in part due to decreased Dicer expression and a resultant decrease in mature miRNA expression.

The data presented herein as well as that of others [11, 23] suggests that high Dicer levels in breast cancer are associated with a well-differentiated epithelial, ERα+ phenotype, while lower Dicer levels are found in the less differentiated ERα- cells. Furthermore, Dicer has been shown to be positively regulated by estradiol [1]. Our findings demonstrate that while the majority of miRNAs are more abundant in ERα+ cells, miR-221/222 and miR-29a are striking exceptions. ESR1 is directly targeted by miR-221/222 and miR-22 [52, 65], and we demonstrate that these miRNAs cooperate to decrease ERα. In clinical samples, we find that expression of miR-222 and ERα is mutually exclusive, consistent with previous reports of miR-222 repressing ESR1 [48, 65] and the reciprocal negative regulatory loop whereby ERα represses miR-222 [16].

We show that miR-221/222 and miR-29a directly target Dicer, and these miRNAs are likely responsible for repressing Dicer expression and function in ERα breast cancer. We find that let-7 is higher in ERα breast cancer cells, and it also directly targets and represses Dicer1 [19, 60]. Since there are let-7 sites in close proximity to both the miR-221/222 and miR-29a binding sites, it is possible that these miRNAs work cooperatively.

MiR-200c represses a program of mesenchymal genes to maintain an epithelial state [6, 14, 15, 22, 25, 34], and here, we show that it also positively regulates Dicer, likely
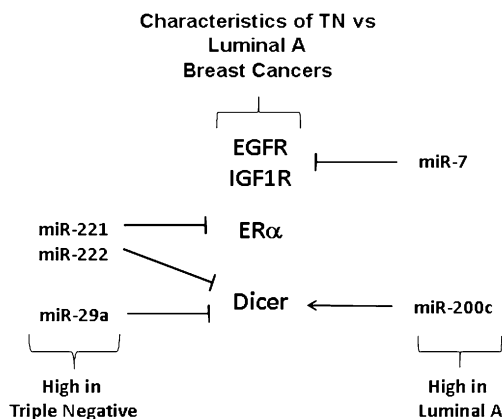

Fig. 7 MiRNA regulation of key proteins in luminal and triple negative breast cancers. Dicer and ERα are expressed at high levels in luminal breast cancers. MiR-221 and miR-222 are high in TN breast cancers and target both Dicer and ERα. MiR-29a is also high in TN breast cancers and targets Dicer. MiR-200c is high in luminal breast cancers and increases Dicer expression. EGFR, IGF1R, and IRS-2 are often activated or overexpressed in TN cancers and are all targeted by miR-7

through an indirect, yet to be identified mechanism. Importantly, this may represent an additional means by which miR-200c promotes a well-differentiated epithelial phenotype. Our studies indicate that a subset of miRNAs may be low due to insufficient Dicer. While lower levels of mature miRNAs in ERα− cells can be explained by decreased Dicer levels, this begs the question as to how some miRNAs (such as miR-221/222 and miR-29a) are abundant in ERα− breast cancers in the face of low Dicer expression. In lower organisms, such as *Drosophila*, there are two Dicer proteins; however, in humans, only one Dicer gene exists (*Dicer1*). Not all miRNAs are equally affected by Dicer depletion [21, 35], suggesting either that miRNA stability is a factor, or perhaps another enzyme exists that can process certain miRNA precursors when Dicer levels are low. For example, miR-451 can be fully processed by Ago2 [9, 13], which is higher in ERα− breast cancers [11] and personal communication (Dorraya El-Ashry and Phillip Miller).

Since both *Dicer* and *ESR1* and their protein products are low or absent in TN breast cancer cells, it makes sense that both are targeted by miRNAs abundant in TN cells. However, both the *ESR1* and *Dicer* 3′ UTRs also have putative target sites for miRNAs that are highly expressed in ERα+ cells. It is likely that other factors are interfering with the miRNA–mRNA interaction. For instance, RNA-binding proteins can bind 3′ UTRs and prevent or recruit miRNA binding [3, 4, 29, 30] or target sites can be mutated or absent due to shortening of the 3′ UTR [20, 46, 50, 55]. Non-coding RNAs or pseudogenes can act as decoys to soak up miRNAs and prevent them from interacting with a target [54, 61]. For instance miR-193b (5.5-fold higher in ESR1+ cells in our study) directly targets *ESR1* when it is transfected into MCF7 cells [40]. Perhaps overexpression of this miRNA can overcome whatever is preventing the already abundant endogenous miR-193b from targeting *ESR1*. Similarly, miR-103/107 was recently reported to directly target *Dicer1* [44]. However, in our study and others [42, 45], miR103/107 is higher in ERα+ cells (which have high Dicer) as compared to ERα- cells. While miR-193b and miR-103/107 can target ESR1 and Dicer if overexpressed, these miRNA are already expressed at higher levels in ERα+ cells that express substantial Dicer. Nevertheless, it is possible that these miRNAs naturally fluctuate under certain conditions in order to fine tune or limit ERα or Dicer protein levels.

We sought to determine if any of the miRNAs differentially expressed in ERα+ versus negative breast cancer cells are differentially expressed because they are regulated by estradiol-bound ERα [2, 8, 32]. Several miRNAs located in the miR-17-92 cluster or its paralog clusters are upregulated by estrogen. The miR-17-92 cluster (also known as *oncomir-1*) has been implicated in several types of cancers [12, 17,

51]. MiR-7 was also both estrogen regulated and more abundant in ERα+ cells. MiR-7 targets EGFR and decreases proliferation [41, 62]. We further demonstrate that miR-7 can also reduce IGF1R and IRS-2 protein expression. EGFR and IGF1R are often overexpressed and constitutively active in TN breast cancers and contribute to an aggressive phenotype [33, 38]. Similarly, IGF1R is often activated in aggressive cancers with poor prognosis, and overexpression of IGF1R in a mouse model results in mammary gland tumors with a basal-like phenotype [31]. Since IRS-2 is a signaling intermediate in the IGF1R pathway [49, 53], miR-7 could be a very effective means by which to abrogate this pathway. Our data suggest that effective re-introduction of miR-7 into TN breast cancer could offer an advantage over inhibitors targeting either EGFR or IGF1R since it would target both pathways simultaneously.

In summary, we demonstrate that the most highly differentially expressed miRNAs more abundant in ERα− breast cancers, namely miR-221/222 and miR-29a, directly repress *Dicer1*. In contrast, miR-200c, which is more abundant in ERα+ breast cancer cells, increases Dicer protein levels. We conclude that miRNAs differentially expressed in ERα+ versus negative breast cancer cells function to control some of the most distinguishing characteristics of the luminal A as compared to TN breast cancer subtypes such as ERα status, Dicer protein levels, and EGFR and IGF1R growth factor receptor expression.

Conflict of Interest The authors declare that they have no conflict of interest.

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Research Article

Loss of miR-200c: A Marker of Aggressiveness and Chemoresistance in Female Reproductive Cancers

Dawn R. Cochrane, Erin N. Howe, Nicole S. Spoelstra, and Jennifer K. Richer

Department of Pathology, Denver School of Medicine, University of Colorado, Aurora CO, 80045, USA

Correspondence should be addressed to Jennifer K. Richer, jennifer.richer@ucdenver.edu

We focus on unique roles of miR-200c in breast, ovarian, and endometrial cancers. Members of the miR-200 family target ZEB1, a transcription factor which represses E-cadherin and other genes involved in polarity. We demonstrate that the double negative feedback loop between miR-200c and ZEB1 is functional in some, but not all cell lines. Restoration of miR-200c to aggressive cancer cells causes a decrease in migration and invasion. These effects are independent of E-cadherin status. Additionally, we observe that restoration of miR-200c to ovarian cancer cells causes a decrease in adhesion to laminin. We have previously reported that reintroduction of miR-200c to aggressive cells that lack miR-200c expression restores sensitivity to paclitaxel. We now prove that this ability is a result of direct targeting of class III beta-tubulin (TUBB3). Introduction of a TUBB3 expression construct lacking the miR-200c target site into cells transfected with miR-200c mimic results in no change in sensitivity to paclitaxel. Lastly, we observe a decrease in proliferation in cells transfected with miR-200c mimic, and cells where ZEB1 is knocked down stably, demonstrating that the ability of miR-200c to enhance sensitivity to paclitaxel is not due to an increased proliferation rate.

1. Introduction

Specific miRNAs have been found to be expressed in cell type-specific manner, at specific developmental stages, and in disease states including cancer [1, 2]. During the initiation and progression of cancer, miRNAs have been observed to act as oncogenes or tumor suppressors [3, 4]. While some miRNAs are overexpressed in cancers, the majority appear to be lost and often localize to fragile sites [5]. Differences in the miRNA expression profiles of normal compared to cancerous tissue of the endometrium, breast and ovary have been documented [6–11]. MiRNAs can affect the expression of a large number of proteins, including those involved in pathways relevant to cancer, such as apoptosis, migration and metastasis. Thus, miRNAs hold promise as biomarkers for several types of cancer [12, 13].

Epithelial to mesenchymal transition (EMT) is a normal process that occurs during development in which individual cells or groups of cells become motile. The same process is thought to be used by cancer cells during tumor progression to enable them to become more motile and thus more metastatic [14]. EMT involves reprogramming of the cells by transcription factors such as ZEB1, SIP1 (ZEB2), Twist, Snail, and Slug [15]. A hallmark of EMT is loss of E-cadherin expression, loss of polarity, acquisition of mesenchymal markers, and increased motility [16, 17]. Both ZEB1 and the closely related ZEB2 bind E-box like sequences in the E-cadherin promoter, recruit the corepressor CtBP and thereby repress E-cadherin [18]. ZEB1 also represses additional genes involved in polarity [16]. ZEB1 expression is confined to cells of mesenchymal origin, while normal epithelial cells and low grade carcinomas do not express ZEB1. However, we and others have shown that in high grade, aggressive carcinomas that have undergone EMT, ZEB1 can be expressed, leading to loss of E-cadherin [19–22].

Several miRNAs have been implicated in the process of EMT, among them are the members miR-200 family [23–25]. This family contains five members (miR-200a, -200b, -200c, -141 and, -429) which are highly homologous. Originally, miR-200c was reported to directly bind ZEB1 and cause degradation of the mRNA, resulting in an upregulation of E-cadherin [26]. Subsequently, other reports have shown

that all members of the miR-200 family, since they share a high degree of homology especially in their seed sequence, are capable of repressing both ZEB1 and ZEB2 [27–29]. We have demonstrated that miR-200c represses not only ZEB1/2, but a program of transcripts normally expressed only in cells of mesenchymal origin [30]. Since members of the miR-200 family are responsible for repressing ZEB1 and ZEB2 as well as other mesenchymal genes, these miRNAs are considered "guardians of the epithelial phenotype." MiR-200 family members are therefore thought to be expressed in an epithelial cell-specific manner in normal tissues. Recently, the ability of ZEB1 to transcriptionally repress expression of miR-200 family members has been documented [31, 32]. This double negative feedback loop between miR-200 family members and ZEB1 allows for plasticity between the epithelial and mesenchymal states [33].

In this paper, we focus on the role of miR-200c in breast, ovarian, and endometrial cancers. The mutual repression between ZEB1 and miR-200c is functional in some, but not all cells that we have tested. Increasing miR-200c levels causes a decrease in adhesion to laminin. We demonstrate that the decrease in migration and invasion observed when miR-200c is reintroduced to cancer cells that lack it is independent of restoration of E-cadherin. Lastly, we have previously demonstrated that class III beta tubulin (TUBB3) is directly controlled by miR-200c. Expression of TUBB3 is known to be a common mechanism of resistance to microtubule-targeting agents in many types of cancer. Here, we present conclusive data that repression of TUBB3 is the mechanism whereby miR-200c restores sensitivity to paclitaxel. Taken together, these data demonstrate that loss of miR-200c is a marker for chemoresistance and aggressiveness in breast, ovarian, and endometrial cancers.

2. Materials and Methods

2.1. Cell Culture. Hec50 cells, representing the more aggressive Type II endometrial cancers [19], were cultured in DMEM with 10% FBS, and 2 mM L-glutamine. MDA-MB-231 are a triple negative breast cancer cell line and were grown in media containing 5% FBS, HEPES, nonessential amino acids, L-glutamine, penicillin, streptomycin, and insulin. Hey cells were grown in RPMI with 5% FBS. All cells were grown in a 37°C incubator with 5% CO_2. The identity of all the cell lines was confirmed by DNA profiling using the Identifiler Kit (Applied Biosystems).

2.2. Transfections. Lipofectamine 2000 (Invitrogen) was combined with pre-200c (miRNA mimic) or scrambled negative control (Ambion) at a concentration of 60 nM and incubated in serum free RPMI for 20 minutes prior to addition to Hey cells. Cells were incubated at 37°C for 4 hours before replacement of FBS to 10%. Protein and RNA were harvested 48 hours posttransfection.

TUBB3 (from Fernando Cabral, University of Texas - Houston Medical School) was cloned into pCI-neo. Transient transfection of 3.3 μg of TUBB3 plasmid or empty vector (pCI-neo, Promega) per well in a 6-well plate was performed using lipofectamine 2000.

2.3. Generation of Stable Cell Lines. Transduction of cells was performed using SMARTvectorTM shRNA Lentivral Particles (Thermo Scientific Dharmacon). Each cell line was transduced with 3 separate lentiviral constructs targeting ZEB1 as well as two controls: SMARTvector Empty Vector particles and SMARTvector Firefly Luciferase Control particles. The former is a negative control and does not correlate with gene silencing and the latter is a positive control targeting firefly luciferase plasmids PGL2 and PGL3. All vectors are packaged and contain a TurboGFP and an SCMV promoter, as well as a puromycin-resistance selectable marker.

MDA231 and Hec50 cells were plated at 3000 cells/well and 1500 cells/well, respectively, in triplicate using 96 well plates. The following day, media was replaced with 80 μl of fresh media containing 10 μg/mL polybrene (Sigma). The amount of viral particles/well was determined using the following calculation: (MOI × CN)/VT, where MOI (multiplicity of infection) = 10 TU/cell, CN = number of cells/well, and VT = stock viral titer of 10^4 TU/μL. Viral particles were added in a total volume of 20 μL to each well. The following day, transduction media was removed and wells were rinsed with PBS and replaced with regular media. Once confluent, cells were trypsinized and replated in 48 well plates. At this point, antibiotic selection was initiated and cells were ultimately expanded and maintained using 1 μg/mL of puromycin (Sigma).

2.4. Immunoblotting. Whole cell protein extracts were denatured and 50 μg separated on 8% SDS PAGE gels and transferred to PVDF membranes. The membranes were blocked in 5% milk in TBS-T, and then probed overnight at 4°C. Primary antibodies were diluted in 5% milk in TBS-T. The primary antibodies used were ZEB1 (rabbit polyclonal from Dr. Doug Darling, University of Kentucky, 1 : 1500 dilution), E-cadherin (clone NCH-38 from DAKO, 1 μg/mL), TUBB3 (rabbit polyclonal PRB-435P from Covance, 1 : 5000 dilution), and α-tubulin (clone B-5-1-2 from Sigma, 1 : 20000 dilution). After incubation with appropriate HRP-conjugated secondary antibody, bands were detected using Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer).

2.5. Real Time RT-PCR. RNA was harvested from cells using Trizol (Invitrogen) as per the manufacturer's instructions. Prior to generating cDNA, mRNA was treated with DNase1 (Invitrogen) for 15 minutes at room temperature. RNA was reverse transcribed into cDNA in a reaction containing reaction buffer, dNTPs, RNAse inhibitor (Applied Biosystems), random hexamers, and 200 U of MuLV-RT (Applied Biosystems). The reaction proceeded at 22°C for 10 minutes, then at 37°C for one hour. For normalization, real time RT-PCR was performed on the cDNA using eukaryotic 18S rRNA endogenous control primers and FAM-MGB probe (Applied Biosystems). TaqMan MicroRNA Reverse Transcription kit was used to generate cDNA for real time RT-PCR reaction in conjunction with a miR-200c specific primer and probe (ABI, assay ID 002300). The reverse transcription primer for miR-200c is a hairpin primer which is specific for the mature miRNA and will not bind to the

precursor molecules. Reported values are the means and standard errors of 3 biological replicates.

The relative mRNA or miRNA levels were calculated using the comparative Ct method ($\Delta\Delta$Ct). Briefly, the cycle threshold (Ct) values for the rRNA were subtracted from Ct values of the target gene to achieve the ΔCt value. The $2^{-\Delta Ct}$ was calculated for each sample and then each of the values was then divided by a control sample to achieve the relative miRNA levels ($\Delta\Delta$Ct).

2.6. Migration and Invasion Assays. The assays were performed on MDA-MB-231 stable empty vector or shZEB1 #2, or Hey cells transiently transfected with the miR-200c mimic for 48 hours. Cells were serum starved for 12 hours prior to performing the assay. BD BioCoat Control Insert Chambers 24-well plate with 8 micron pore size and BD BioCoat Matrigel Invasion Chambers were used for migration and invasion assays, respectively. After starvation, cells were removed from the plate and 50000 Hey cells or 250000 MDA-MB-231 cells were plated in 0.5 mL media with 0.5% FBS in the upper chamber. In the lower chamber 0.8 mL of 50% conditioned media plus 50% complete media containing an additional 10% FBS was used as an attractant. Hey cells were incubated for 24 hours and MDA-MB-231 cells for 48 hours at 37°C. Migrating or invading cells on the lower surface of the membranes were stained with Diff-Quik stain (Fisher) and counted manually using ImagePro Plus software (Mediacybernetics Inc.).

2.7. Adhesion Assays. Adhesion assays were performed using InnoCyte ECM Cell Adhesion Assays (Calbiochem) for Collagen IV, Fibronectin, Basement Membrane Complex and Laminin. To each well 50000 cells were added and the plates were incubated at 37°C for 1.5 hour. The wells were gently washed with PBS before adding the Calcein-AM solution. The plates were incubated for 1 hour at 37°C, and fluorescence was read with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The relative fluorescent units were plotted, and the error bars represent standard error of the mean over four replicates.

2.8. Clonogenic Assay. Hey cells were plated into 6-well plates at a density of 2000 cells per well. Twenty-four hours after plating, the cells were treated with 0, 1, 2, 3, 4, or 5 nM paclitaxel (Sigma) in triplicate. The cells were incubated at 37°C for 8 days before fixing and staining with crystal violet. Photos were taken of the plates and the images analyzed using ImageJ software (NIH). The average number of colonies and the average total area was plotted, with error bars representing the standard error of the mean over the three replicates.

2.9. Cell Death ELISAs. Hec50 cells were transfected with the miR-200c mimic as described previously for the Hey cells. Twenty-four hours after transfection, cells were treated with 0, 15, 20 or 25 nM paclitaxel (Sigma). Twenty-four hours after treatment, the Cell Death ELISA (Roche) which recognizes mono- and oligonucleosomes in the cytoplasm of dying cells was performed as per manufacturer's instructions.

2.10. Proliferation Assays. Cells were plated into 6-well plates (4000 Hey cells/well and 6000 MDA-MB-231 or Hec50 cells/well). At time points indicated, cells were trypsinized and counted using the Vi-cell Cell Viability Counter (Beckman Coulter).

3. Results

3.1. Reciprocal Repression of miR-200c and ZEB1 Occurs in Some but Not All Cell Types. Just as members of the miR-200 family can repress ZEB1 by degradation of its transcript, ZEB1 can repress expression of the miR-200 family members by binding to E-boxes within their promoter regions [31, 32]; see Figure 1(a). Directly increasing miR-200c levels in Hey cells (aggressive serous ovarian cell line) by transfection of a miR-200c mimic (pre-200c) results in repression of ZEB1 expression (Figure 1(b)). Although ZEB1 is a repressor of E-cadherin, we did not observe E-cadherin expression induced by the repression of ZEB1 in these cells (data not shown). However there are several other mechanisms through which E-cadherin can be lost including methylation of the promoter [34, 35] and chromosomal deletion [36, 37]. In contrast, we have previously shown that transient transfection of the miR-200c mimic into MDA-MB-231 (an aggressive triple negative breast cancer cell line) and Hec50 (an aggressive Type 1 endometrial cancer cell line) causes a marked repression of ZEB1 and a restoration of E-cadherin expression [30]. Presently, we stably transduced lentiviral shRNAs targeting ZEB1 into these two cell lines (Hec50 and MDA-MB-231). While two of the shRNAs did not decrease ZEB1 protein, shRNA #2 caused an almost complete repression of ZEB1 expression resulting in re-expression of E-cadherin in both cell lines (Figures 2(c) and 2(d)). Intriguingly, while knock down of ZEB1 in MDA-MB-231 cells causes the expected increase in miR-200c levels (indicative of the reciprocal regulation), no such increase is observed in Hec50 cells. This suggests that while reciprocal repression of miR-200c and ZEB1 occurs in some cell lines, it does not occur in all.

3.2. Restoration of miR-200c to Aggressive Cancer Cells Results in Decreased Migration, Invasion, and Cell Adhesion. It has been previously shown that the miR-200 family members cause a decrease in cell migration and invasion [27, 29, 30]. We observe a decrease in migration and invasion in the MDA-MB-231 cells in which ZEB1 has been knocked down, resulting in an increase in miR-200c levels. In the MDA-MB-231 cells there is 52% decrease in migration and a 50% decrease in invasion in the shZEB1 #2 containing cells in which ZEB1 is completely knocked down versus luciferase control (Figures 2(a) and 2(b)). We show here that the same holds true in the aggressive ovarian cancer Hey cell line. This cell line is highly migratory and invasive, and reintroduction of miR-200c to these cells results in an 83% decrease in migration and a 79–86% decrease in invasion compared to negative controls (Figures 2(c) and 2(d)). However, it is interesting to note that the effect on migration and invasion caused by miR-200c is independent of the E-cadherin status of the cells, since unlike the MDA-MB-231 cells, Hey cells
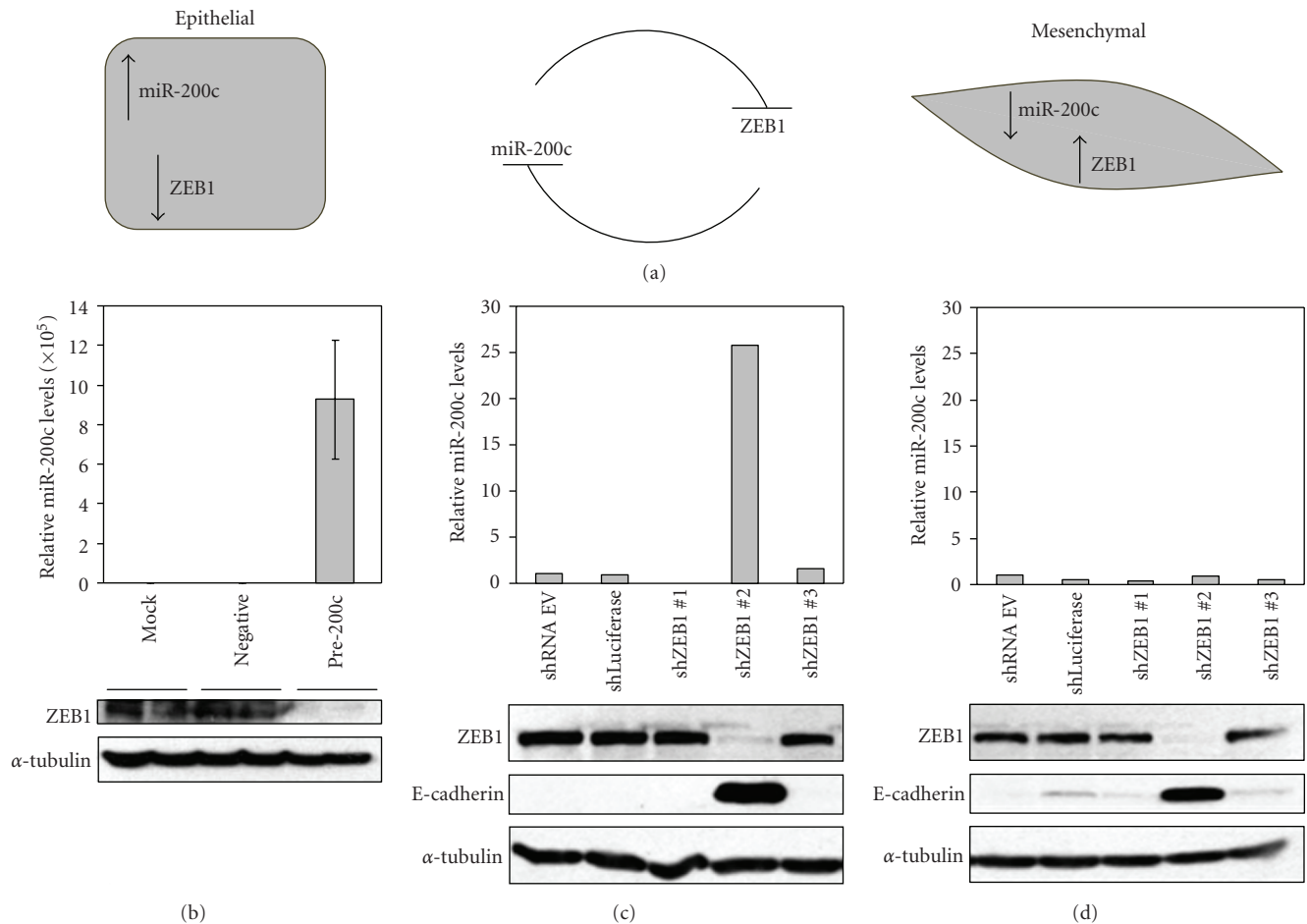
Figure 1: Reciprocal repression between ZEB1 and miR-200c occurs in some, but not all cell types. (a) Schematic of the mutual repression of ZEB1 and miR-200c. (b) Western blotting for ZEB1 and α-tubulin loading control, and real time RT-PCR for miR-200c in Hey ovarian cells transiently transfected with a miR-200c mimic. Real time RT-PCR for miR-200c and western blotting for ZEB1, E-cadherin, and α-tubulin in MDA-MB-231 breast cancer cells (c), and Hec50 endometrial cancer cells (d) stably transfected with shRNA lentiviral vector targeting ZEB1 (shZEB), luciferase (shLuciferase), or the empty vector (shRNA EV).

do not regain E-cadherin expression in response to decreased ZEB1 levels.

While E-cadherin protein affects epithelial cell-cell contact, we also wished to determine if miR-200c affects adhesion to substrates as measured by fluorescent adhesion assays. Hey cells transiently transfected with the miR-200c mimic showed a small but statistically significant decrease in adhesion to basement membrane complex (BMC) and laminin (Figures 3(a) and 3(b)). There is also a trend towards decreased adhesion to collagen IV (Figure 3(c)); however, this did not reach statistical significance. No difference in adhesion to fibronectin was observed (Figure 3(d)). Since there was an affect on adhesion to BMC and laminin in ovarian cancer cells with high miR-200c levels, we performed the adhesion assay with the Hec50 and MDA-MB-231 cells in which ZEB1 had been stably knocked down. We again see a decrease in adhesion to BMC and laminin in the MDA-MB-231 cells; however, only the decrease in BMC binding is statistically significant (Figures 3(e) and 3(f)). In contrast

to the Hey and MDA-MB-231 cells, there was no decrease in adhesion to either substrate in the Hec50 cells in which ZEB1 is knocked down (data not shown), but there is not a concomitant increase in miR-200c, as shown in Figure 1(d). This result suggests that the effects on adhesion may be mediated through miR-200c.

3.3. Increased Chemosensitivity to Paclitaxel with miR-200c Expression. We have previously demonstrated that miR-200c expression causes increased chemosensitivity to microtubule targeting agents such as paclitaxel. While the ELISA cell death assay that we have used previously to demonstrate this property of miR-200c is a short-term assay, we confirm here, in a relatively long-term clonogenic assay, that there is increased sensitivity of Hey cells to paclitaxel when transfected with pre-200c (Figure 4(a)). We observe a 49–55% decrease in total area and a 67–70% decrease in the number of colonies in the pre-200c treated cells versus the negative control with 5 nM paclitaxel treatment (Figures 4(b)
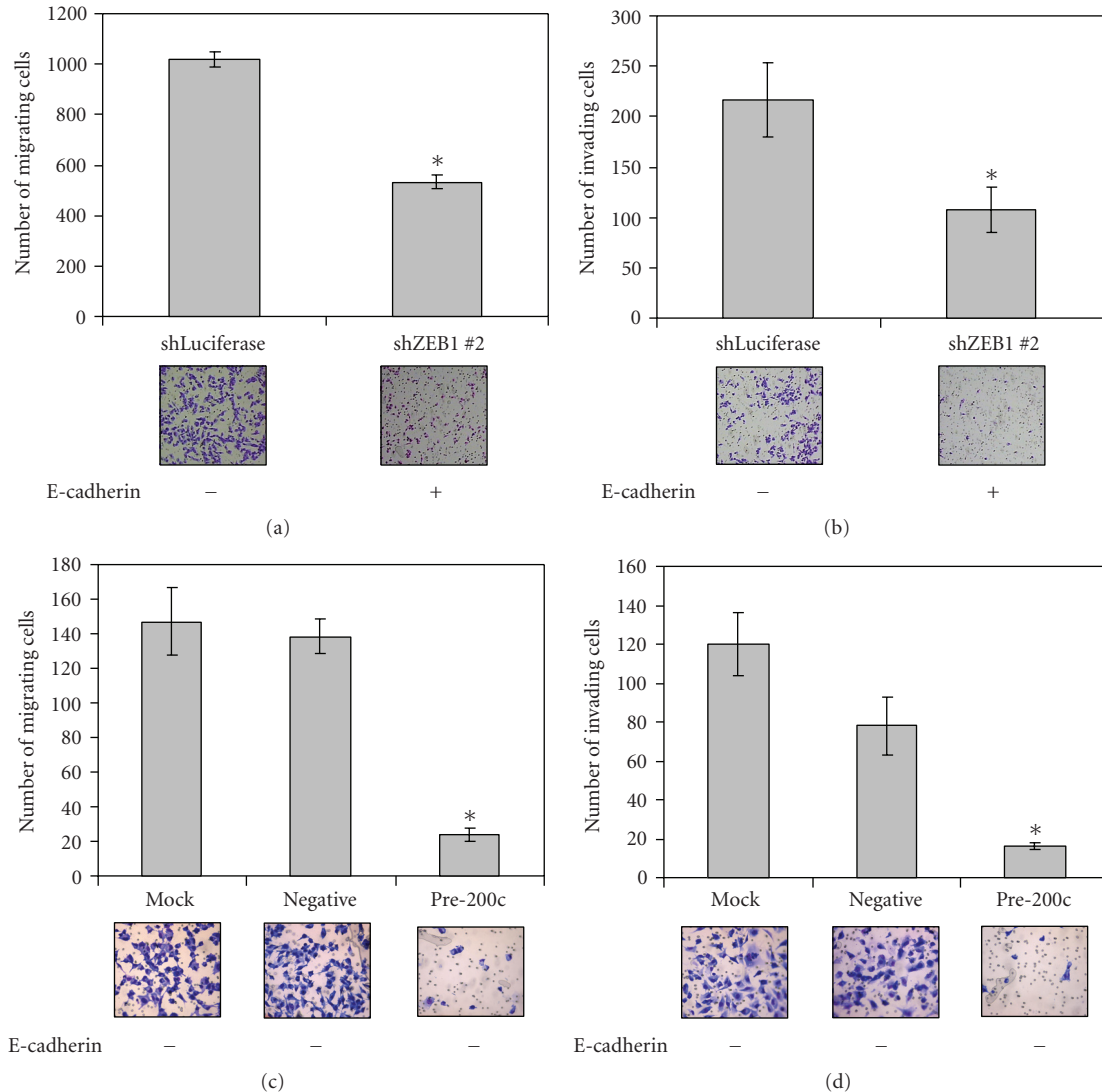
(a)

(b)

(c)

(d)

FIGURE 2: Increased miR-200c decreases migration and invasion, not necessarily dependent on restoration of E-cadherin. Migration (a) and invasion (b) assays for MDA-MB-231 cells stably expressing an shRNA targeting ZEB1 with representative images below. Migration (c) and invasion (d) assays in Hey cells transiently transfected with a miR-200c mimic. Asterisks indicate a statistically significant difference ($P < .05$, Student's t-test) versus negative controls.

and 4(c)). As the assay is conducted over a relatively long period of time, the maximum dose of paclitaxel used is relatively small compared to what is used in the assays that look at acute toxicity (i.e., 24 hours). At doses of paclitaxel of 10 nM and higher, no colonies are formed in the assay (data not shown).

We have previously implicated the ability of miR-200c to directly target *TUBB3* (class III beta tubulin) as being the mechanism responsible for the increased chemosensitivity to microtubule targeting agents. TUBB3 is normally only expressed in neuronal cells; however aberrant expression of TUBB3 in several different types of cancers has been shown to cause resistance to paclitaxel [38–43]. We demonstrated that miR-200c directly targets *TUBB3* for degradation. To definitively test whether TUBB3 is responsible for the miR-200c-mediated increase in chemosensitivity to paclitaxel, we

transfected cells with a TUBB3 construct lacking its 3' UTR (containing the miR-200c binding site) which is therefore not able to be targeted by miR-200c. Transfection of this exogenous TUBB3 construct does not affect the transfection of the miR-200c mimic, nor its ability to downregulate ZEB1 and upregulate E-cadherin (Figures 5(a) and 5(b)) in Hec50 cells. When the Hec50 cells are transfected with an empty vector (no exogenous TUBB3) in addition to miR-200c mimic, there is a statistically significant increase in sensitivity to paclitaxel as measured in a cell death ELISA; see Figure 5(c). However, when the cells are transfected with the TUBB3 expression vector lacking its 3' UTR, the enhanced sensitivity to paclitaxel is lost; see Figure 5(d). Therefore, expression of exogenous TUBB3 lacking the miR-200c target site reverses the chemosensitivity to paclitaxel caused by increased miR-200c expression.
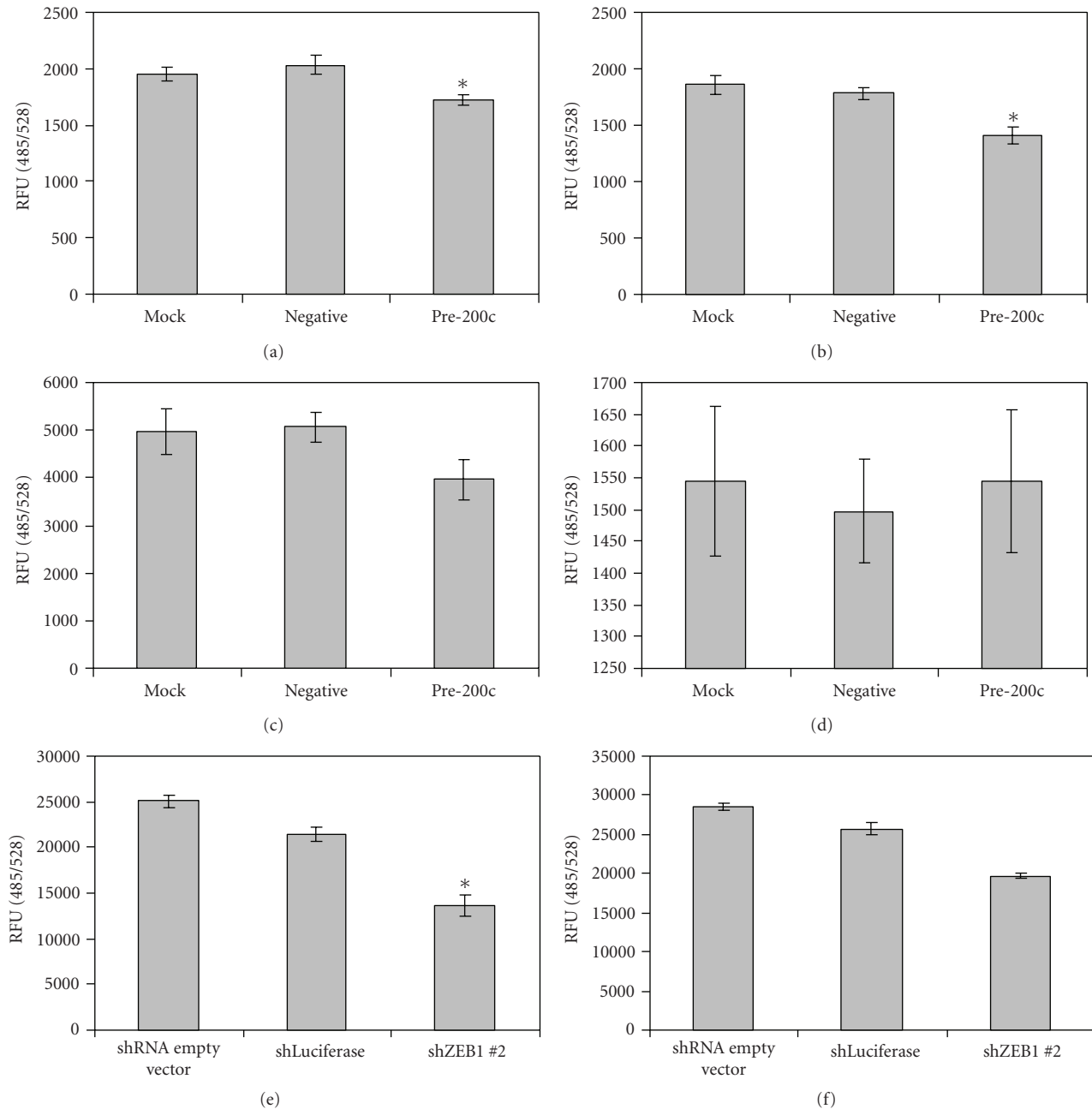
FIGURE 3: Increased miR-200c levels decrease adhesion to substrates. Fluorescent adhesion assays of Hey cells to (a) basement membrane complex, (b) laminin, (c) collagen type IV, and (d) fibronectin. Adhesion of MDA-MB-231 cells to (e) basement membrane complex and (f) laminin. Asterisks indicate a statistically significant difference ($P < .05$, Student's t-test) versus the negative controls.

It can be argued that cells with increased proliferation would be more sensitive to microtubule poisons and that could be an alternative explanation for the observed chemosensitivity upon restoration of miR-200c. We therefore performed proliferation assays in the three cell types and found decreased proliferation in all three (Figures 6(a), 6(b), and 6(c)). Since the decrease in proliferation is observed in all three cell types, including the Hec50s where there was no increase in miR-200c levels, it is likely that the effects on proliferation occur via ZEB1 and not by miR-200c.

The fact that the increase in chemosensitivity is found in cells that are proliferating more slowly than the negative controls demonstrates that increased proliferation is not the mechanism behind the increase in chemosensitivity.

4. Discussion

In this paper we build on our previous work to further characterize the role that loss of miR-200c plays in generating an aggressive cancer phenotype. We focus on ovarian,
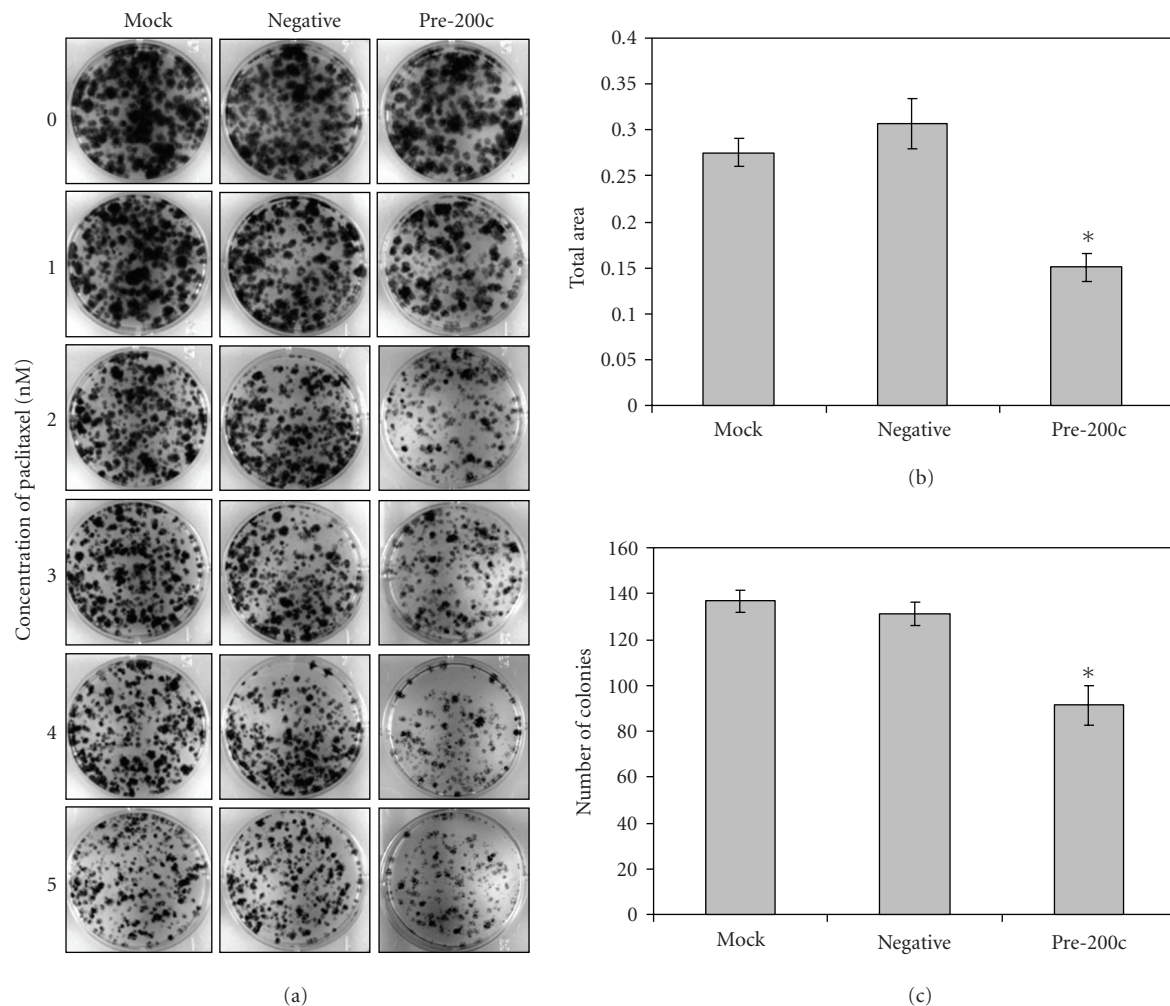
(a)

(b)

(c)

FIGURE 4: Chemosensitivity to paclitaxel induced by miR-200c expression. (a) Clonogenic assay in Hey cells either mock transfected, transiently transfected with a negative control, or miR-200c mimic and treated with 0–5 nM paclitaxel. The total area (b) and number of colonies (c) at 5 nM paclitaxel are quantified. Asterisks indicate statistically significant difference ($P < .05$, Student's t-test) versus negative controls.

endometrial, and breast cancer. The miR-200 family is crucial for the maintenance of the epithelial phenotype. ZEB1 is normally only expressed in cells of mesenchymal origin; however, its aberrant expression is observed in cancers that have undergone EMT. ZEB1 (and the closely related ZEB2) transcripts are targeted by miR-200c and the other miR-200 family members. Interaction of any of the miR-200 family members with the ZEB transcripts results in degradation and inhibition of translation. Therefore the maintenance of miR-200c expression in normal epithelial cells serves to prevent ZEB1 and ZEB2 from being expressed. Since both ZEB1 and ZEB2 repress genes involved in polarity, repression of these proteins serves to maintain polarity, an important epithelial cell characteristic. We have recently shown that in addition to repressing ZEB1 and 2, miR-200c represses a program of transcripts normally only expressed in cells of mesenchymal and neuronal origin, such as fibronectin (FN1), neurotrophic tyrosine kinase (NTRK2), quaking 1 (QKI), and TUBB3 [30]. Thus, miR-200c maintains epithelial cell characteristics

not only by maintaining polarity via repression of ZEB1 and ZEB2, but also by repressing additional non-epithelial genes.

It has been recently demonstrated that miR-200c and ZEB1 regulate each other in a double-negative feedback loop [31, 32]. The miR-200 family of miRNAs is expressed in two clusters, one on choromosome 1p36.33 and the other on chromosome 12p12.31. E-boxes are located in the promoter region of each of these clusters. ZEB1 can bind these E-boxes and directly repress all miR-200 family members [31]. Therefore, in cells that have undergone EMT, ZEB1 and 2 not only serve to repress genes involved in polarity, but also repress the miR-200 family and thereby release the repression of many genes characteristic of the mesenchymal phenotype. Central to the double feedback loop between miR-200c and ZEB1 is TGF-β. During TGF-β-induced EMT, there is an increase in Ets1 which binds to and activates the promoter of ZEB1 [44]. Therefore, in a tumor microenvironment, increased TGF-β levels are thought to result in an increase of ZEB1 transcription to a point where it can overcome the
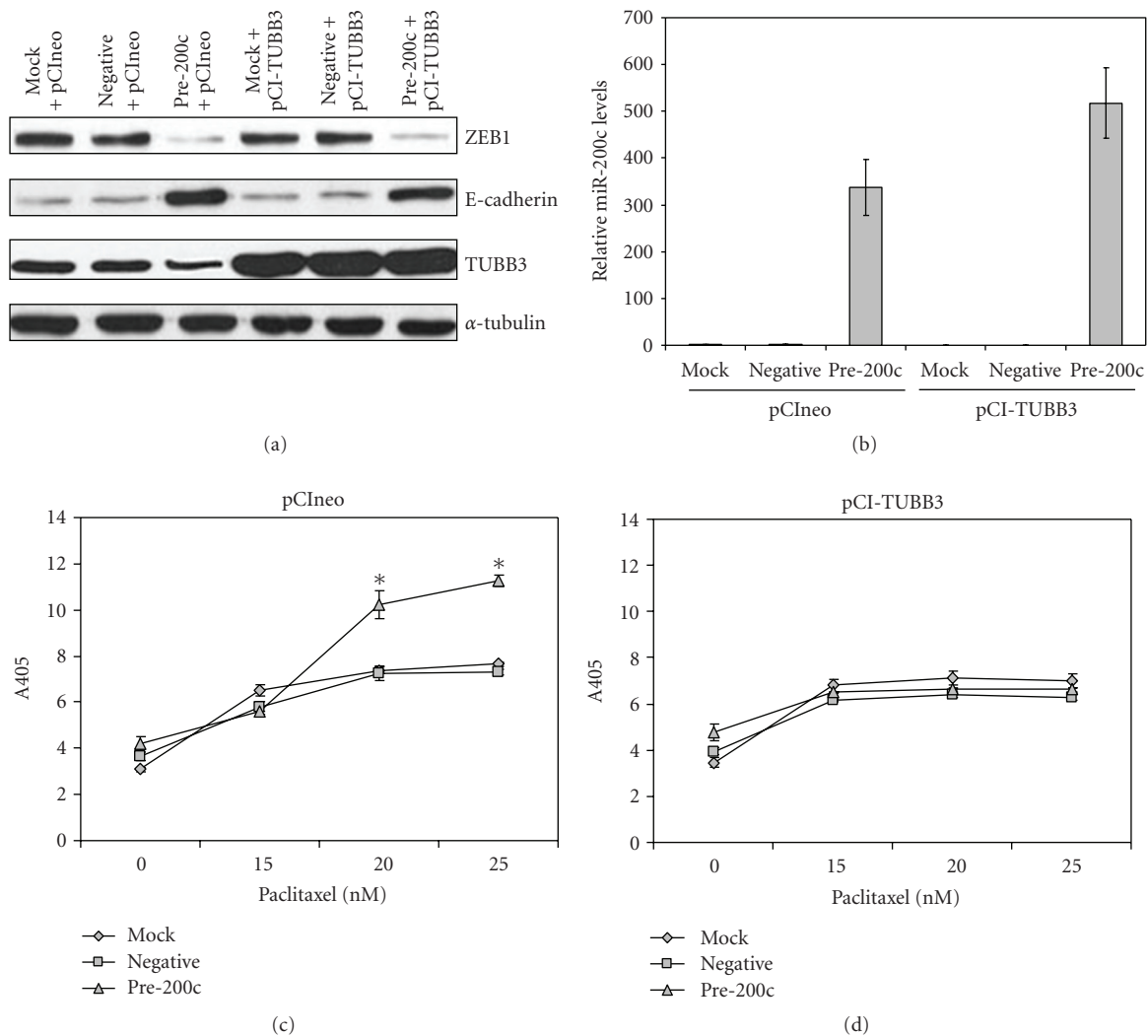
(a)

(b)

(c)

(d)

FIGURE 5: Restoration of TUBB3 reverses miR-200c-mediated enhanced chemosensitivity to paclitaxel. (a) Western blots for ZEB1, E-cadherin, TUBB3, and α-tubulin in Hec50 cells transiently transfected with a miR-200c mimic or negative controls and an expression vector for TUBB3 or empty vector. (b) Real time RT-PCR for miR-200c. Cell death ELISA for cells transfected with a miR-200c mimic and an empty vector (c) or TUBB3 expression vector (d) treated with various concentrations of paclitaxel. Asterisks indicate statistically significant difference ($P < .05$, Student's t-test) versus negative controls.

repression caused by miR-200c. As ZEB1 protein begins to be made, it can then repress the miR-200 family members, resulting in progression through EMT [32].

We have previously shown that restoration of miR-200c in Hec50 endometrial cells and MDA-MB-231 breast cancer cells causes repression of ZEB1 and re-expression of E-cadherin protein. Here we show that transfection of miR-200c mimic into Hey cells, an aggressive serous ovarian cell line, also causes a dramatic repression of ZEB1; however no expression of E-cadherin was observed. To test whether the double-negative feedback loop is intact in the Hec50 and MDA-MB-231 cells, these cells were infected with lentivirus expressing an shRNA against ZEB1. In both cell lines, efficient knock down of ZEB1 was achieved, as was re-expression of E-cadherin. In MDA-MB-231 cells, ZEB1 knock down resulted in an increase in miR-200c levels,

as would be expected from the negative feedback loop. However, this was not the case in Hec50 cells, where there was no increase in miR-200c. Whether the break in the negative feedback loop is an anomaly of this particular cell line remains to be tested. The mechanism behind the phenomenon is also unknown; however, it does offer an opportunity to dissect the contribution of ZEB1 versus that of miR-200c to the phenotype of the cells. For example, significantly decreased proliferation was observed in the Hey cells transiently transfected with the miR-200c mimic as well as in the MDA-MB-231 and Hec50 cells that have ZEB1 stably knocked down, although miR-200c levels did not rise in the Hec50s. Therefore it is likely that the decrease in proliferation is due to the lack of ZEB1, not an increase in miR-200c. Conversely, the decrease in adhesion to the basement membrane complex and laminin was only
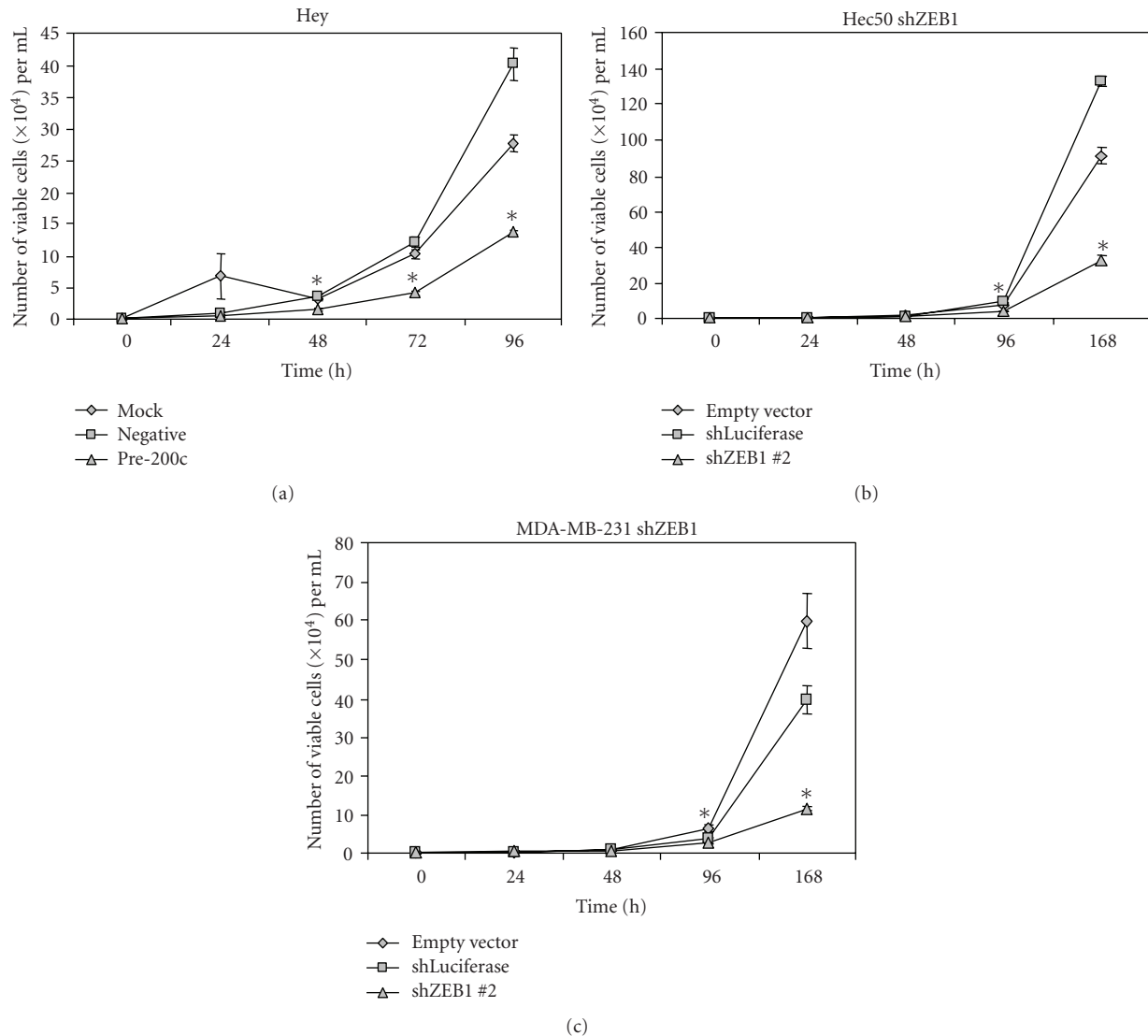
FIGURE 6: Proliferation assay in Hey cells transiently transfected with a miR-200c mimic (a), or Hec50 cells (b), and MDA-MB-231 cells (c) stably expressing an shRNA against ZEB1. Asterisks indicate statistically significant difference ($P < .05$, Student's t-test) versus negative controls.

observed in the Hey and MDA-MB-231 cells, and not the Hec50s, suggesting that this phenotype is a function of miR-200c expression rather than ZEB1.

We and others have previously shown that restoration of miR-200c to cancer cells that do not express it causes a decrease in invasion and migration [27, 30]. Here we show that knock down of ZEB1 in MDA-MB-231 cells, which causes an increase in miR-200c, negatively affects migration and invasion. Furthermore, we show that restoration of miR-200c in Hey ovarian cancer cells results in a dramatic decrease in migration and invasion even though E-cadherin is not restored in these cells, despite complete repression of ZEB1. Loss of E-cadherin expression can result from mechanisms other than ZEB1 transcription repression, including chromosomal deletion and promoter hypermethylation [34–37]. Possibilities for its continued

absence in these cells include promoter methylation such that even when repression by ZEB1 is relieved, E-cadherin will not be expressed, or perhaps levels of another transcriptional repressor such as Snail or Twist remain high and repress E-cadherin. Regardless of the mechanism, the effects of miR-200c on invasion and migration appear to be independent of E-cadherin status. While E-cadherin is involved in epithelial cell-cell adhesion and its expression has been shown to negatively affect migration and invasion [45, 46], increased miR-200c is able to decrease migration and invasion on its own. We have previously observed that restoration of miR-200c affects genes involved in cell motility and invasion such as ARHGDIB, NTRK2, EPHB1, and FN1 [30].

We demonstrate that miR-200c causes a decrease in adhesion to basement membrane complex, laminin, and perhaps collagen type IV. This observation is particularly

relevant to ovarian cancer because the cancerous cells adhere to sites within the peritoneal cavity. During the progression of cancer there is switching of the expression patterns of the cell surface adhesion molecules, such as the cadherins and integrins [47–49]. Although the change in the number of adherent cells appears modest, this might play a significant role in developing a potential treatment for ovarian cancer. The ability of ovarian cancer cells to spread and adhere to the peritoneal cavity is one of the major phenotypes of this disease. A small change in ability of the cells to adhere might reflect a great decrease in the tumor burden and/or increase the ability to debulk the tumor. These results are independent of E-cadherin expression since the decrease in adhesion is observed in both the MDA-MB-231 cells (where E-cadherin expression is regained with increased miR-200c) and in Hey cells, where it is not.

Clonogenic assays reveal that there is an increase in chemosensitivity to paclitaxel with increased miR-200c levels. Indeed, acquired resistance to paclitaxel in ovarian cancer cells has been shown to be associated with EMT, resulting in an aggressive phenotype [50]. Clinically, aberrant expression of TUBB3 (not normally expressed in epithelial cells) has been found to be associated with resistance to taxanes [38–41]. We have previously shown that TUBB3 is a direct target of miR-200c and suggested that its repression by miR-200c is the mechanism behind the ability of miR-200c to increase chemosensitivity to microtubule targeting agents [30]. Here, we perform the definitive experiment to prove that miR-200c-mediated TUBB3 downregulation is indeed the cause of the enhanced chemosensitivity. We utilized exogenous TUBB3 lacking its 3'UTR such that it cannot be targeted by miR-200c and show that resistance to paclitaxel is maintained even in the presence of miR-200c. In contrast, endogenous TUBB3 is reduced when miR-200c is added, resulting in enhanced chemosensitivity to paclitaxel.

Microtubule targeting agents such as paclitaxel work more efficiently in cells that are rapidly dividing. Consequently, it could be argued that the increase in chemosensitivity caused by miR-200c is due to increased proliferation. However, we show that increase of miR-200c or direct knockdown of ZEB1 results in decreased proliferation in three different types of cancer cells. It is therefore the downregulation of TUBB3, not an increase in proliferation that is responsible for the enhanced chemosensitivity to taxanes observed with restoration of miR-200c to resistant cancer cells.

5. Conclusions

MiR-200c expression serves to maintain the epithelial phenotype in well-differentiated, low-grade, breast, ovarian, and endometrial cancer cells. This phenotype includes decreased adhesion to laminin and decreased migration and invasion. Furthermore we find that not all of miR-200c's actions can be attributed to the restoration of E-cadherin via targeting of ZEB1. We further prove that miR-200c-mediated repression of TUBB3 is the cause of enhanced chemosensitivity to microtubule targeting agents. Lastly we demonstrate that not

all cells exhibit the double negative feedback loop between miR-200c and ZEB1 and that this can be exploited to identify the distinct roles of miR-200c as compared to ZEB1.

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MicroRNA-200c mitigates invasiveness and restores sensitivity to microtubule-targeting chemotherapeutic agents

Dawn R. Cochrane, Nicole S. Spoelstra, Erin N. Howe, Steven K. Nordeen, and Jennifer K. Richer

Department of Pathology, University of Colorado Denver School of Medicine, Aurora, Colorado

Abstract

The transcription factor ZEB1 is normally not expressed in epithelial cells. When inappropriately expressed in carcinomas, ZEB1 initiates epithelial to mesenchymal transition due to its ability to repress E-cadherin and other genes involved in polarity. Recently, ZEB1 and ZEB2 have been identified as direct targets of the microRNA-200c family. We find that miR-200c levels are high in well-differentiated endometrial, breast, and ovarian cancer cell lines, but extremely low in poorly differentiated cancer cells. Low or absent miR-200c results in aberrant expression of ZEB1 and consequent repression of E-cadherin. Reinstatement of miR-200c to such cells restores E-cadherin and dramatically reduces migration and invasion. Microarray profiling reveals that in addition to ZEB1 and ZEB2, other mesenchymal genes (such as *FN1, NTRK2*, and *QKI*), which are also predicted direct targets of miR-200c, are indeed inhibited by addition of exogenous miR-200c. One such gene, class III β-tubulin (*TUBB3*), which encodes a tubulin isotype normally found only in neuronal cells, is a direct target of miR-200c. This finding is of particular significance because we show that restoration of miR-200c increases sensitivity to microtubule-targeting agents by 85%. Because expression of TUBB3 is a common mechanism of resistance to microtubule-binding chemotherapeutic agents in many types of solid tumors, the ability of miR-200c to restore chemosensitivity to such agents may be explained by its ability to reduce TUBB3. Because miR-200c is crucial for maintenance of epithelial identity, behavior, and sensitivity to chemotherapy, we propose that it warrants further investigation as a therapeutic strategy for aggressive, drug-resistant cancers. [Mol Cancer Ther 2009;8(5):1055–66]

Requests for reprints: Jennifer K. Richer, Department of Pathology, University of Colorado Denver, Mail Stop 8104, P.O. Box 6511, Aurora, CO 80045. Phone: 303-724-3735; Fax: 303-724-3712. E-mail: Jennifer.Richer@ucdenver.edu

Introduction

We previously reported that the transcription factor ZEB1 (zinc finger E-box binding homeobox 1; also known as TCF8, ZFHX1A, ZFHEP, AREB6, BZP, NIL-2-A, and δEF1) is aberrantly expressed in type 2 endometrial cancers that have undergone an epithelial to mesenchymal transition (EMT; refs. 1, 2). ZEB1 binds to E-box like sequences (CACCTG) and is involved in the development of mesodermal and neural tissues. ZEB1 and ZEB2 (SIP1) play a role in EMT during tumor progression by directly repressing E-cadherin (3–5) and other epithelial markers (6, 7). Endometrial cancers can be divided into two subtypes. Type 1 endometrial cancers (low-grade endometrioid adenocarcinomas) retain many epithelial characteristics and are relatively nonaggressive. In contrast, type 2 endometrial cancers are a heterogeneous group of poorly differentiated tumors (International Federation of Gynecology and Obstetrics grade 3 endometrioid adenocarcinomas, serous papillary, clear cell, and malignant mixed Müllerian tumors) with advanced stage at diagnosis and poor prognosis as compared with type 1 tumors (8, 9). Type 2 tumors often have lost epithelial markers and gained mesenchymal characteristics, and this affects their clinical behavior (aggressiveness). Some have suggested that a similar classification for ovarian cancers would be useful (10–12).

Recent research has implicated microRNAs (miRNA), acting as oncogenes and tumor suppressors, in the development and progression of cancers (13, 14). Many miRNAs localize to fragile sites and are frequently lost in cancer (15). Recently, it was reported that miR-200c targets *ZEB1* (16). It was later shown that other members of the miR-200 family, which share sequence homology, can target both *ZEB1* and the closely related gene, *ZEB2* (17, 18). These recent data suggest that the miR-200 family is responsible for maintenance of the epithelial phenotype, at least partially via repression of *ZEB1* and *ZEB2*. Indeed, we show that miR-200c expression is strongly associated with a more benign, less aggressive phenotype in endometrial, ovarian, and breast cancer cell lines. Levels inversely correlate with ZEB1 and positively correlate with E-cadherin. We show that restoration of miR-200c expression to cancer cells that lack it suppresses ZEB1 expression, thereby completely

restoring E-cadherin. We find that reinstatement of miR-200c leads to a dramatic decrease in cell migration and invasion and an up to 85% increase in sensitivity to micro-tubule-targeting chemotherapeutic agents. We suspected that, just as miR-200c indirectly maintains E-cadherin expression by directly repressing ZEB1 and ZEB2, it also likely targets other genes involved in polarity, migratory capacity, and chemosensitivity. Consequently, we performed expression profiling to identify additional genes altered by restoration of miR-200c to cancer cells. Indeed, we find that miR-200c inhibits a program of mesenchymal genes, in addition to ZEBs. We show that one such gene, class III β-tubulin (*TUBB3*), a microtubule component normally found only in neuronal cells, is a direct target of miR-200c. Because expression of TUBB3 is a prevalent mechanism of resistance to microtubule-binding chemotherapeutic agents in many solid tumors, the fact that TUBB3 is reduced by miR-200c may account for the dramatic effect of restoration of miR-200c expression on sensitivity to this clinically important class of chemotherapeutic agents.

Materials and Methods
Cell Culture
Hec50 cells, which well represent the more aggressive type 2 endometrial cancers (19), were cultured in DMEM with 10% fetal bovine serum (FBS) and 2 mmol/L L-glutamine. AN3CA cells (American Type Culture Collection), derived from a grade 3 endometrioid adenocarcinoma (also an aggressive form of endometrial cancer and thought to represent the characteristics of type 2 endometrial cancers), and Ishikawa cells (representing a low-grade endometrioid adenocarcinoma, or type 1 tumor; ref. 19) were grown in MEM with 5% FBS, nonessential amino acids, and 1 nmol/L insulin. EEC B37 cells are normal endometrial epithelial cells that have been immortalized with hTERT (20) and were maintained in F12 MEM containing 10% FBS, 2 mmol/L L-glutamine, and 160 ng/mL insulin. HIESC are normal endometrial stromal cells transformed with SV40 large T antigen (21) and were grown in RPMI with 10% FBS, penicillin, streptomycin, and sodium pyruvate. MCF-7 and T47D breast cancer cells were grown in DMEM, 10% FBS, and L-glutamine. BT-474 cells were grown as above, with the addition of nonessential amino acids and insulin. MDA-MB-231 and ZR75 cells were grown in medium containing 5% FBS, HEPES, nonessential amino acids, L-glutamine, penicillin, streptomycin, and insulin. BT-549 cells were grown in RPMI supplemented with 10% FBS and insulin. MCF-7, T47D, ZR75, and BT-474 are all relatively well-differentiated breast cancer cell lines that retain estrogen receptors and the epithelial marker E-cadherin. In contrast, MDA-MB-231 and BT-549 cells represent less differentiated breast cancers negative for estrogen receptors and E-cadherin. All of the ovarian cell lines (2008, Hey, SKOV3, OVCA 420, and OVCA 433) were grown in RPMI with 10% FBS. All cells were grown in a 37°C incubator with 5% CO_2. The identity of all the cell lines was confirmed by DNA profiling using the Identifiler Kit from Applied Biosystems.

Immunoblotting
Whole-cell protein extracts were denatured, and 50 μg were separated on 8% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. After blocking in 5% milk in TBS-T, membranes were probed overnight at 4°C. Primary antibodies used include ZEB1 (rabbit polyclonal from Dr. Doug Darling, University of Louisville, Louisville, KY; 1:1,500 dilution), E-cadherin (clone NCH-38 from DAKO; 1 μg/mL), N-cadherin (clone 13A9 from Upstate; 1:5,000 dilution), vimentin (clone V9 from Sigma; 1:2,000 dilution), TUBB3 (clone SDL.3D10 from Sigma; 1:400 dilution), PSTAIR (rabbit polyclonal from Upstate; 1 μg/mL), and α-tubulin (clone B-5-1-2 from Sigma; 1:15,000 dilution). After incubation with appropriate secondary antibody, results were detected using Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer).

Real-time Reverse Transcription-PCR
RNA was harvested from cells using Trizol (Invitrogen). Before generating cDNA, mRNA was treated with DNase 1 (Invitrogen) for 15 min at room temperature. RNA was reverse transcribed into cDNA in a reaction containing reaction buffer, 10 mmol/L DTT, 1 mmol/L deoxynucleotide triphosphates, RNase inhibitor (Promega), random hexamers (250 ng), and 200 units of MULV-RT (ABI). The reaction proceeded at 25°C for 10 min, then at 37°C for 1 h. For normalization, real-time reverse transcription-PCR (RT-PCR) was done on the cDNA using eukaryotic 18S rRNA endogenous control primers and FAM-MGB probe (ABI). TaqMan MicroRNA Reverse Transcription kit was used to generate cDNA for real-time RT-PCR reaction in conjunction with a miR-200c–specific primer and probe (ABI, assay ID 002300). The reverse transcription primer for miR-200c is a hairpin primer that is specific for the mature miRNA and will not bind to the precursor molecules. Reported values are the means and SEs of three or four biological replicates, as indicated. For validation of the microarray data, SYBR Green real-time RT-PCR was done using primers specific for *CHK2* (forward 5′-GCTCTTGGCTGTGCAGATTA-3′, reverse 5′-ACGGTTA-TACCCAGCAGTCC-3′), *ARHGDIB* (forward 5′-CTGGGT-CCCTCTTCAACACT-3′, reverse 5′-TGTTCTAGGGAC-CACGTTGA-3′), *MAL2* (forward 5′-GCAGCCACTCCT-GAGTGATA-3′, reverse 5′-CGTAAAGCCAGACCCA-AACT-3′), *EPHB1* (forward 5′-GTGAGATGGACAGCTC-CAGA-3′, reverse 5′-ACGATCCCATAGCTCCAAAC-3′), *LEPR* (forward 5′-ATTGGAGCAATCCAGCCTAC-3′, reverse 5′-CAGGGGCTTCCAAAGTAAAG-3′), *ST6GAL-NAC5* (forward 5′-TGAGCTCTTCAAGCAGGAGA-3′, reverse 5′-CATTGTAAACCAGCCAGTGC-3′), and *TUBB3* (forward 5′-CGAAGCCAGCAGTGTCTAAA-3′, reverse 5′-GGAGGACGAGGCCATAAATA-3′). To avoid the possibility of amplification artifacts, the PCR products for all SYBR Green primer pairs were verified by gel electrophoresis to be single products.

The relative mRNA or miRNA levels were calculated using the comparative C_t method ($\Delta\Delta C_t$). Briefly, the C_t (cycle threshold) values for the rRNA or actin were subtracted from C_t values of the target gene to achieve the
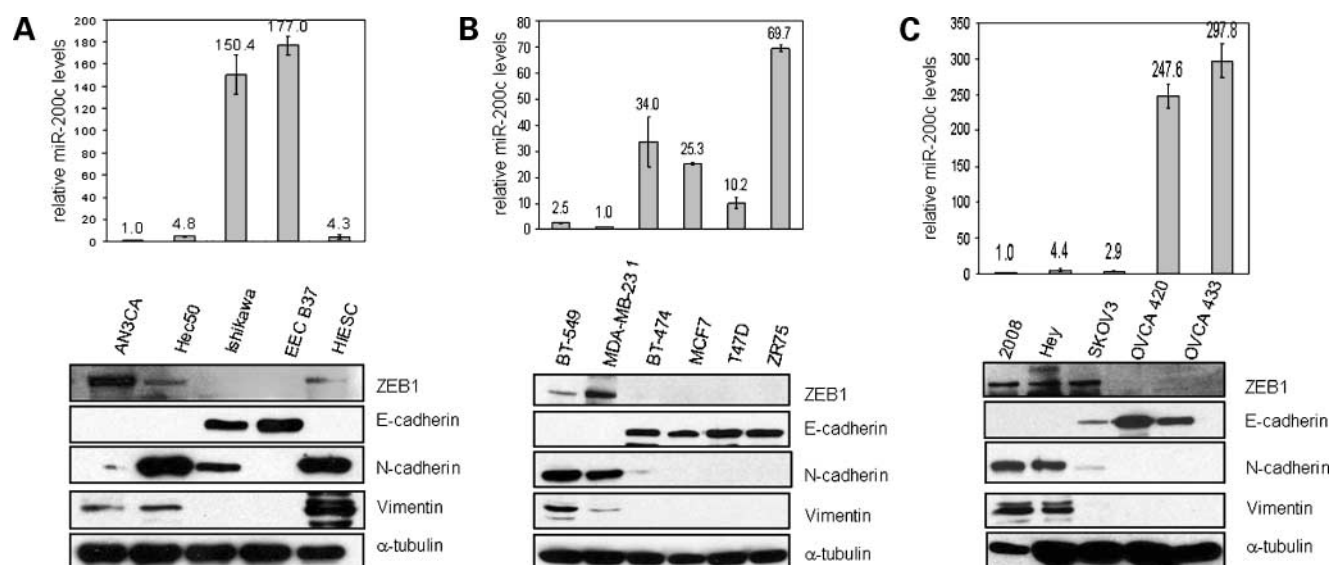
Figure 1. MiR-200c and ZEB1 are inversely correlated in endometrial, breast, and ovarian cancer cells. **A,** RNA and protein were harvested from endometrial cancer cell lines—AN3CA and Hec50 (high-grade, representing type 2 endometrial cancers), and Ishikawa (representing type 1 endometrial cancer), EEC B37 (hTERT transformed normal endometrial epithelial cells), and HIESC (SV40 transformed normal endometrial stromal cells). RNA was assayed for miR-200c by real-time PCR (*top*). Immunoblots of whole-cell protein extracts were probed for ZEB1, E-cadherin, N-cadherin, vimentin, and α-tubulin as a loading control (*bottom*). **B,** RNA and protein were harvested from aggressive breast cancer cell lines (BT-549 and MDA 231) as well as the more differentiated cell lines (BT-474, MCF7, T47D, and ZR75) for detection of miR-200c and immunoblot analysis of epithelial and mesenchymal markers. **C,** ovarian cell lines (2008, Hey, SKOV3, OVCA 420, and OVCA 433) were harvested and assayed as above. Each graph is representative of three independent experiments. For real-time RT-PCR, each column represents the mean of quadruplicate samples and bars represent SE. MiR-200c levels are normalized to rRNA and are relative to AN3CA, MDA-MB-231, or 2008 cells, respectively.

ΔC_t value. The $2^{-\Delta C_t}$ was calculated for each sample and then each of the values was then divided by a control sample to achieve the relative mRNA or miRNA levels ($\Delta \Delta C_t$).

Transfection, Migration, and Invasion Assays

Lipofectamine 2000 (Invitrogen) was incubated with pre-200c (miRNA mimic) or scrambled negative control (Ambion) at a concentration of 60 nmol/L incubated in serum-free DMEM for 20 min before addition to Hec50 cells. Cells were incubated at 37°C for 4 h before replacement of FBS to 10%. Protein and RNA were harvested 48 h after transfection. For wound healing assay, 24 h after transfection, cells were trypsinized and plated into six-well dishes in triplicate at high density. The next day, a wound was made through the cells using a p200 tip. Pictures were taken immediately and then 4, 8, 12, and 24 h later. For migration and invasion assays, 36 h after transfection, cells were serum starved for 12 h before performing the assay. BD Bio-Coat Control Insert Chambers 24-well plate with 8-μm pore size and BD BioCoat Matrigel Invasion Chambers were used for migration and invasion assays, respectively. After starvation, cells were trypsinized and 2.5×10^4 cells were plated in 0.5 mL MEM with 0.5% FBS in the upper chamber. In the lower chamber, 0.8 mL of 50% conditioned medium from Hec50 cells plus 50% DMEM with 10% FBS and L-glutamine was used as an attractant. Cells were incubated for 48 h at 37°C. Migrating or invading cells on the lower surface of the membranes were stained with Diff-Quik stain (Fisher) and counted manually using ImagePro Plus software (Mediacybernetics, Inc.).

Fluorescent Immunocytochemistry

Cells were grown on glass coverslips, rinsed with PBS, and fixed with 10% neutral buffered formalin for 5 min, followed by 50% ethanol for 4 min. Coverslips were rinsed again with PBS and stored dry at −20°C. Before staining, coverslips were thawed at room temperature and rinsed with TBS-T (0.05%). ZEB1 antibody was used at 1:1,000 dilution and E-cadherin at 1:50. Staining was done as described previously (1).

Cell Death ELISAs

Hec50 cells were transfected as described above. Twenty-four hours after transfection, cells were treated with 0, 5, 10, 15, 20, or 25 nmol/L of paclitaxel (Sigma) or with 0, 20, 30, 40, or 50 of μmol/L cisplatin [*cis*-diamminedichlorido-platinum(II); Sigma]. In separate experiments, Hec50 (endometrial cancer), MDA-MB-231 (breast cancer), and Hey (ovarian cancer) cell lines were treated with tumor necrosis factor–related apoptosis-inducing ligand (TRAIL;R&D Systems) at 50 ng/mL, FasL (Axxora Life Science, Inc.) at 1 μg/mL, doxorubicin (Calbiochem) at 6 μg/mL, mitomycin C (Sigma) at 100 nmol/L, vincristine (Sigma) at 100 nmol/L, or epothilone B (Sigma) at 100 nmol/L. Twenty-four hours after treatment, the Cell Death ELISA (Roche), which recognizes mononucleosomes and oligonucleosomes in the cytoplasm of dying cells, was done as per manufacturer's instructions.

Statistical Analysis

For the real-time PCR, cell death ELISA, and luciferase assays, a Student's paired *t* test was done to determine
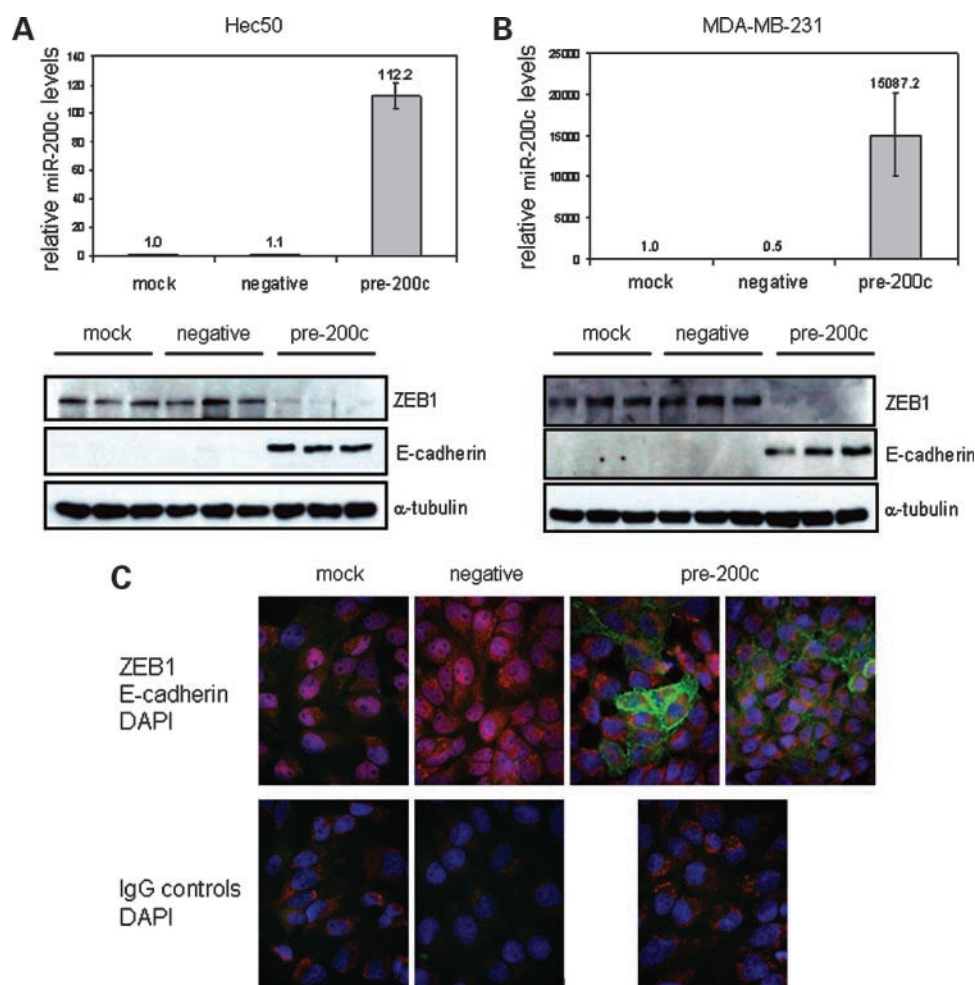
Figure 2. Addition of exogenous miR-200c results in repression of ZEB1 and restoration of E-cadherin protein. Hec50 endometrial cancer cells (**A**) and MDA-MB-231 breast cancer cells (**B**) were treated with transfection reagent only (*mock*), scrambled negative control mimic (*negative*), or miR-200c mimic (*pre-200c*). After 48 h, RNA was harvested and miR-200c levels were determined by real-time PCR (*top*). *Columns,* mean of quadruplicate samples; *bars,* SE. The miR-200c levels are normalized to rRNA and are relative to mock transfection levels. Western blots of protein from the three experimental groups (mock, negative, or miR-200c treated) were probed for ZEB1, E-cadherin, and α-tubulin as a loading control. Three replicates per treatment group are shown. For both real-time RT-PCR and Western blots, results are representative of one of three independent experiments. **C,** Hec50 cells grown on coverslips were treated as above and fluorescent immunocytochemistry results using antibodies recognizing ZEB1 (*red*), E-cadherin (*green*), and 4′,6-diamidino-2-phenylindole (*DAPI; blue*) are shown merged. *Bottom,* relevant IgG-negative controls. Magnification, ×1,000.

statistical significance (Microsoft Excel). Values were considered to be statistically significant if $P \leq 0.05$.

Microarray Analysis

To confirm the integrity of triplicate RNA samples, RNA-nano chips were run on a Bioanalyzer (Agilent). The cDNA was generated and processed according to the GeneChip Expression Analysis Technical Manual (Affymetrix). Labeled complementary RNA was made using the GeneChip_IVT Labeling Kit (Affymetrix), fragmented, and hybridized to HGU133 Plus 2.0 Affymetrix oligonucleotide microarray chips, which contain >54,000 probe sets. GeneSpring GX 9.0 (Agilent) software was used for analysis and clustering of array data. Data were filtered using a 1.5-fold change cutoff and a *P* value of 0.05 (ANOVA, with Benjamini Hochberg FDR multiple testing correction). Ingenuity Pathway Analysis software (Ingenuity Systems) was used to determine which pathways are highly represented among the genes that change in response to miR-200c.

Luciferase Assays

A 262-bp section of the 3′ untranslated region (UTR) of *TUBB3* containing the putative binding site for miR-200c (predicted from the TargetScan database) was amplified by PCR from HeLa genomic DNA using the following primers: *TUBB3* F, 5′-CCACTAGTCGACGAGGAGGAGT-3′, and *TUBB3* R, 5′-CTCAAGCTTGCCTGGAGCTGCA-3′. The fragment was cloned into the 3′-UTR of a firefly luciferase reporter vector (pMIR-REPORT, Ambion) using *Hin*dIII and *Spe*I. To generate the *TUBB* 3′-UTR containing a mutation in the miR-200c binding site, the following primers were used (mutation in bold): TUBB3mutF, 5′-CCTGCA**TCT**TT-TATGGCCT-CG-3′, and TUBB3mutR, 5′-CATAAA**AGA**TG-CAGGAGGGCGGCAAGG-3′. Two PCR products were generated using the primer pairs of TUBB3F with TUBB3-mutR and TUBB3R with TUBB3mutF. These two PCR products were annealed and used as template for a final PCR reaction generated using the TUBB3F and TUBB3R primers. This generated the final product containing the mutated site, which was cloned into pMIR-Report. Hec50 cells (15,000 per well) in a 96-well plate were transfected with the negative control or pre-200c as described above. After 24 h, the firefly reporter plasmid (0.196 µg) and a renilla luciferase normalization plasmid pRL-SV40 (0.004 µg) were introduced using Lipofectamine 2000. Cells were harvested 48 h later for analysis using the Dual Luciferase Reporter assay system (Promega).

Results

Low MiR-200c Expression Strongly Correlates with Lack of E-cadherin Expression and Gain of Mesenchymal Markers Including ZEB1

We sought to determine if there is a negative correlation between miR-200c and ZEB1 in a panel of endometrial cancer cell lines. Hec50 and AN3CA cells, derived from a serous papillary uterine cancer and a grade 3 endometrioid adenocarcinoma respectively, are highly aggressive and are good models of the behavior of type 2 endometrial cancers (19). In contrast, Ishikawa cells are derived from a well-differentiated, less aggressive type 1 endometrial cancer. EEC B37, a cell line derived from normal endometrial epithelial cells (20), and HIESC, derived from normal endometrial stromal cells (21), were also examined. The miR-200c levels were extremely low in the poorly differentiated type 2 endometrial cell lines and the stromal cell line (Fig. 1A). In comparison, the normal endometrial epithelial cell line and the Ishikawa cells had >150-fold higher miR-200c levels. These results suggest that loss of miR-200c expression is associated with poorly differentiated endometrial carcinoma. Stromal cells also express low levels of miR-200c, consistent with our observa-

tion that ZEB1 is expressed in normal endometrial stroma (1, 2). Immunoblot results reveal that ZEB1 protein is present in the type 2 cell lines (AN3CA and Hec50) as well as in normal stromal cells, but normal epithelial cells and the well-differentiated Ishikawa cancer cells lack ZEB1. Because ZEB1 is a potent repressor of E-cadherin, E-cadherin protein is present only in the normal epithelial cells and Ishikawa cells, which express miR-200c robustly and lack ZEB1. More aggressive endometrial cancers often undergo EMT and begin to express stromal markers (1, 2). The normal stromal cells (HIESC) and the more aggressive cancer cell lines, Hec50 and AN3CA, all express vimentin. Only Hec50 and HIESC cells express N-cadherin. In contrast, neither the normal epithelial cells nor the Ishikawa cells express vimentin and the normal cells lack expression of N-cadherin. We observe a similar negative correlation between miR-200c and ZEB1 expression and a positive correlation between miR-200c and E-cadherin in a panel of breast (Fig. 1B) and ovarian (Fig. 1C) cancer cells.

Restoration of MiR-200c Restores E-cadherin Expression and Reduces Migration and Invasion

To determine if miR-200c controls ZEB1 expression in endometrial cancer cells, we used a commercially available
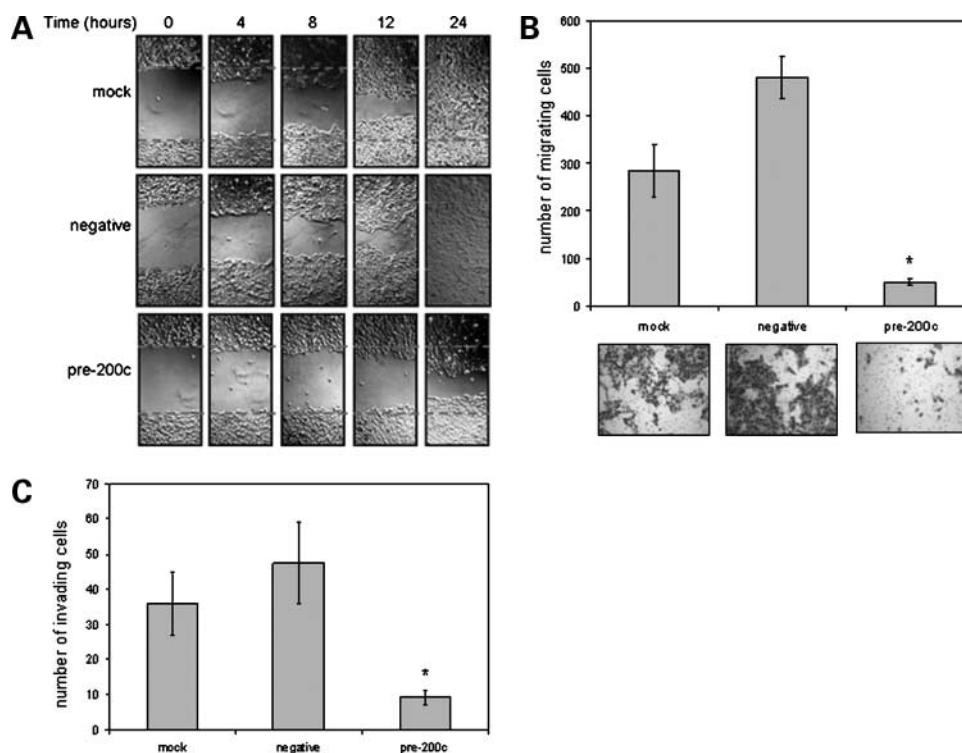

Figure 3. Restoration of miR-200c expression in Hec50 decreases migration and invasion. **A,** Hec50 cells were treated with transfection reagent only, a scramble negative control, or miR-200c mimic. After 48 h, wounds were inflicted and pictures taken at 0, 4, 8, 12, and 24 h after wounding. Lines indicate width of the wound at time zero. Pictures shown are from one experiment representative of three separate experiments (not shown). **B,** mock-, negative-, or miR-200c–transfected cells were subjected to a transwell migration assay. After 48 h, cells on the bottom side of the membrane were stained and mounted onto slides and the mean number of cells in four fields of vision on a cross-hatch was counted with error bars representing SE of four replicates. *, statistically significant difference between the numbers of cells migrating in the pre-200c, compared with either mock-transfected cells or negative control–treated cells [$P = 2.8 \times 10^{-4}$ and $P = 6.0 \times 10^{-8}$, respectively (Student's t test)]. Representative images (×100 magnification) of stained filters are shown. **C,** the number of cells able to invade through Matrigel-coated Boyden chambers was also determined for each group. *Columns,* mean number of cells from four replicates; *bars,* SE. *, statistically significant difference between the pre-200c–treated group, compared with either mock-transfected cells or negative control–treated cells [$P = 0.0039$ and $P = 0.0020$, respectively (Student's t test)].
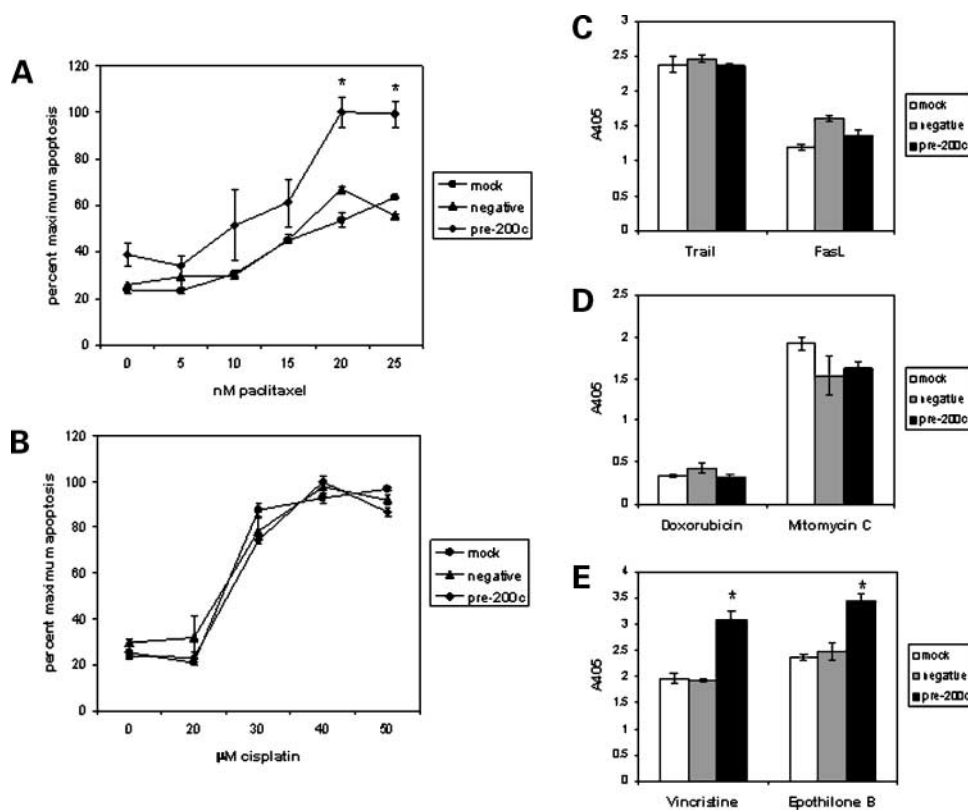
Figure 4. MiR-200c alters cell death in response to microtubule-targeting chemotherapeutic agents specifically. Hec50 cells were treated with transfection reagent only (*mock*), scrambled negative control (*negative*), or miR-200c mimic (*pre-200c*). Twenty-four hours after transfection, cells were treated with 0, 5, 10, 15, 20, or 25 nmol/L of paclitaxel (**A**) or with 0, 20, 30, 40, or 50 μmol/L of cisplatin (**B**) and, 24 h after drug treatment apoptosis, were assayed using a Cell Death ELISA. *Points,* percent maximum apoptosis; *bars,* SE of triplicate samples. This experiment was done twice and representative experiments for each drug are shown. *, $P < 0.05$, between pre-200c–treated cells and mock or negative controls (Student's *t* test). Hec50 cells treated with pre-200c or negative controls were treated with agents that cause apoptosis via cell surface receptors (TRAIL, 50 ng/mL or FasL, 125 ng/mL; **C**), DNA damage (doxorubicin, 1 μg/mL or mitomycin C, 6 μg/mL; **D**), or microtubule poisons (vincristine, 100 nmol/L or epothilone B, 100 nmol/L; **E**), and Cell Death ELISAs were done. *Columns,* mean of five replicates; *bars,* SE. The experiment was repeated on three separate occasions with the same result, and a representative experiment is shown. *, $P < 0.05$, between pre-200c and both the mock and negative controls (Student's *t* test).

miR-200c mimic (pre-200c) to restore miR-200c to Hec50 cells. A time course measuring miR-200c following transfection with the mimic showed maximum levels of expression achieved by 24 hours, gradually decreasing over 6 days, while still remaining above control level (data not shown). At 48 hours after transfection, we achieved a 112-fold expression of miR-200c over mock-transfected and scrambled control–containing cells (Fig. 2A, *top*). Importantly, at the concentration used for transient transfections, the levels of miR-200c achieved are comparable to the endogenous miR-200c levels found in the well-differentiated Ishikawa cells (Fig. 1A). Increasing miR-200c causes inhibition of ZEB1 expression and, importantly, a complete restoration of E-cadherin protein expression (Fig. 2A, *bottom*). To ensure that these effects were not cell type specific, we also performed a transient transfection of pre-200c into MDA-MB-231 cells, an aggressive breast cancer cell line (Fig. 2B). As seen in Fig. 2A and B (*bottom*), restoration of miR-200c to the MDA-MB-231 cells also causes an almost complete repression of ZEB1 levels, resulting in a dramatic appearance of E-cadherin expression. Dual fluorescence immunocyto-

chemistry done on coverslips from the Hec50 transfection experiment show that whereas no E-cadherin (*green*) is observed in the negative control and mock-transfected cells, there is a low level of E-cadherin expression in the majority of the pre-200c–treated cells and very high expression in some areas (Fig. 2C), as would be expected in a transient transfection. There is a decrease in nuclear ZEB1 staining (red, or pink when overlaid with 4′,6-diamidino-2-phenylindole) in the pre-200c–treated cells. Some apparent ZEB1 staining occurs in the cytoplasm in all treatment groups; however, this is likely nonspecific because it is also observed in the isotype antibody control.

To determine if introducing miR-200c renders Hec50 cells less migratory, a wound healing assay was done on the pre-200c–treated cells as well as the negative controls. Figure 3A shows that Hec50 cells treated with pre-200c are distinctively less migratory than the negative control and mock-transfected cells. Furthermore, a transwell migration assay showed a similar loss of migratory capacity (Fig. 3B). Decreases of 82% and 89% in the number of migrating cells were observed in the pre-200c–treated cells compared with
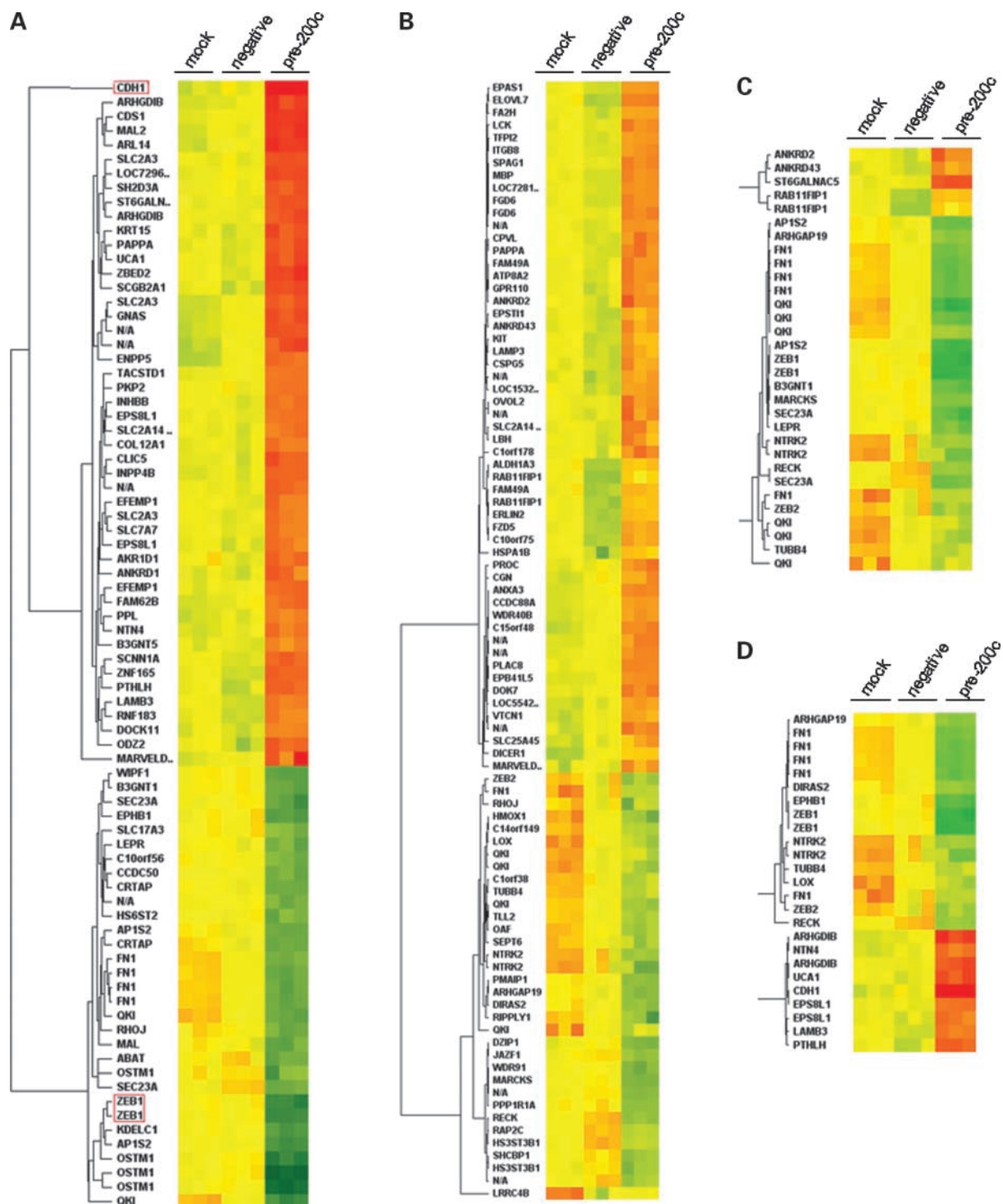
Figure 5. Heatmap of genes significantly affected by restoration of miR-200c levels in Hec50 cells as determined by expression profiling. Hec50 cells were treated in triplicate with mock, negative control, or pre-200c transfection, and gene expression analysis was done on Affymetrix HGU133 Plus 2.0 oligonucleotide cDNA expression array chips. **A,** genes with a statistically significant (ANOVA) ≥1.5-fold up-regulation (*red*) or down-regulation (*green*) in the pre-200c–treated cells versus both the negative control and the mock-transfected cells are shown in a heatmap. Expected alterations in *E-cadherin* (*CDH1*) and *ZEB1* are highlighted. **B,** genes ≥1.5-fold up-regulated or down-regulated in the pre-200c–treated cells versus either negative control are shown in a separate heatmap. **C,** a heatmap of genes from **A** or **B** that are bioinformatically predicted to be targets of miR-200c. **D,** genes differentially regulated by miR-200c implicated in cell migration as determined by Ingenuity Pathway Analysis. Note that each gene was normalized to its average expression over the nine chips, such that the intensities center around 1 and are presented on a scale of −2 to +2 and are thus not indicative of relative fold changes. Fold changes and *P* values for these genes are listed in Supplementary Table S1.[1]

the mock-transfected or negative control–treated cells, respectively. In addition, 81% fewer pre-200c–treated cells were able to invade through Matrigel in a transwell invasion assay as compared with negative control–treated cells (Fig. 3C). MiR-200c did not affect the amount of proliferation of the cells as measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and Hoechst dye assays (data not shown).

Restoration of MiR-200c Enhances Chemosensitivity to Microtubule-Directed Agents

To determine if restoration of miR-200c and the resulting reestablishment of epithelial characteristics would affect chemosensitivity, paclitaxel- and cisplatin-induced apoptosis were measured in cells treated with pre-200c versus controls. A significant increase in chemosensitivity to paclitaxel (Fig. 4A), but not cisplatin (Fig. 4B), was detected in Hec50 cells transfected with miR-200c. The chemosensitivity to 25 nmol/L paclitaxel was increased by 37% and 45% in the cells treated with pre-200c versus the mock and negative controls, respectively. Because paclitaxel and cisplatin have different modes of action, we performed further cell death ELISAs using agents that induce apoptosis via different mechanisms. FasL and TRAIL induce death through tumor necrosis factor–related cell surface receptors in a caspase-8–dependent manner. No increase in chemosensitivity to either TRAIL or

FasL was observed with pre-200c treatment (Fig. 4C). Doxorubicin and mitomycin C, like cisplatin, cause DNA damage that results in apoptosis, and neither agent caused an increase in chemosensitivity in conjunction with pre-200c treatment (Fig. 4D). Epothilone B and vincristine both cause apoptosis by stabilization of microtubules in a mechanism similar to that of paclitaxel. As observed with paclitaxel, pre-200c treatment causes substantial and statistically significant increase in chemosensitivity to these two additional microtubule poisons (Fig. 4E).

Identification of Direct and Downstream Targets of MiR-200c

Whereas it has been previously shown that miR-200c regulates ZEB1 and ZEB2, many other putative miR-200c targets are predicted by bioinformatics based on complementarity, but remained to be validated. To identify miR-200c direct targets or genes downstream of such targets that might be responsible for the reduction in invasiveness and increased sensitivity to microtubule-targeting agents, expression profiling was done on Hec50 cells treated with pre-200c as compared with mock- or scrambled control–transfected cells. Our profiling study identified a cluster of genes significantly differentially regulated by miR-200c as compared with both controls (mock- and scrambled nontargeting miRNA–transfected cells; Fig. 5A). Additional
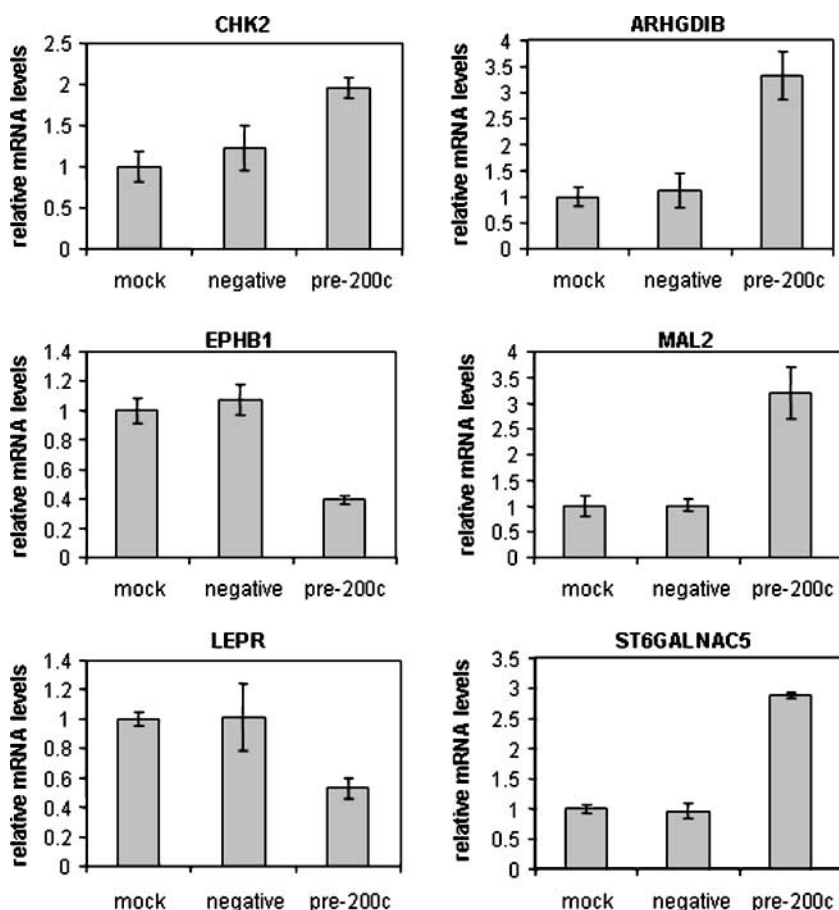

Figure 6. Select genes altered by restoration of miR-200c are validated by RT-PCR. SYBR Green real-time RT-PCR was done on Hec50 cells treated with transfection reagent only (*mock*), 60 nmol/L of a scramble negative control (*negative*), or 60 nmol/L of the miR-200c mimic (*pre-200c*) using primers specific for *CHK2, ARHGDIB, EPHB1, MAL2, LEPR*, and *ST6GALNAC5*. *Bars,* SE of three replicates.
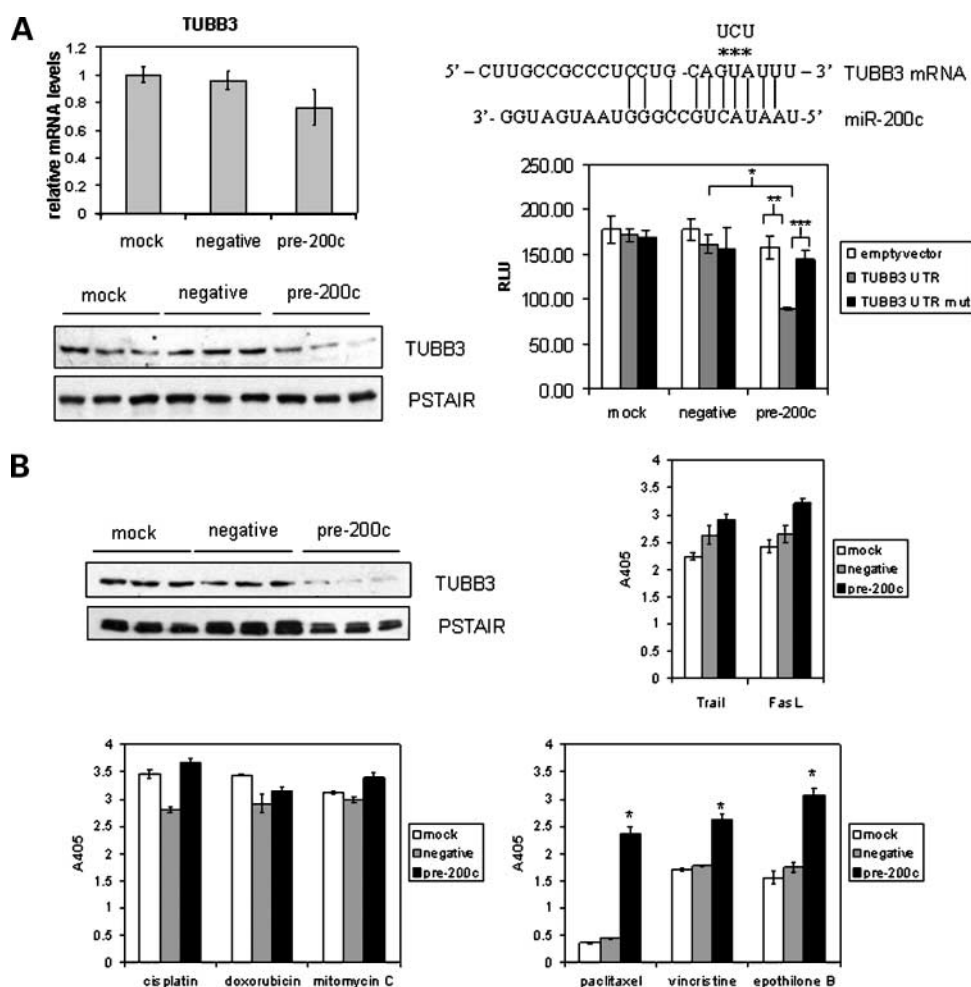
Figure 7. A site in the *TUBB3* 3'-UTR is a direct target of miR-200c, and a decrease in TUBB3 protein corresponds with an increase in cell death in response to microtubule-targeting agents. **A,** SYBR Green real-time RT-PCR using primers specific for *TUBB3* was done on RNA from Hec50 cells treated with miR-200c mimic (*pre-200c*), negative scrambled control (*negative*), or mock-transfected (*mock*) control (*top left*). A corresponding Western blot consisting of protein from the same cells was probed for TUBB3 (*bottom left*) and PSTAIR (used as a loading control). A fragment of the *TUBB3* 3'-UTR (located 117–379 bp after the stop codon) containing the putative miR-200c binding site, or the same fragment with the indicated base pairs that bind to the miR-200c seed sequence mutated, was cloned into the luciferase reporter vector pMIR-REPORT. These constructs, empty pMIR-REPORT vector, vector containing wild-type *TUBB3* 3'-UTR, or mutated *TUBB3* 3'-UTR (*TUBB3* UTR mut), were transfected into Hec50 cells following negative control, pre-200c, or mock transfection. A dual reporter luciferase assay was done, and relative luciferase units (*RLU*) were calculated as firefly luciferase values divided by renilla values. *Columns,* mean of five replicate samples; *bars,* SE. *, $P = 0.041$, statistically significant difference in the amount of luciferase detected when the wild-type *TUBB3* UTR is in the presence of pre-200c versus negative control. **, $P = 0.006$, difference in the amount of luciferase in the presence of pre-200c and either the empty vector or wild-type *TUBB3* 3'-UTR–containing reporter. ***, $P = 0.003$, difference between the amounts of luciferase measured when it is targeted by wild-type versus mutated *TUBB3* 3'-UTR. **B,** an aggressive ovarian cancer cell line, Hey, was treated with pre-200c, a negative control, or mock transfection. *Top left,* Western blot for TUBB3 with PSTAIR as a loading control. A Cell Death ELISA was done on the mock-, negative control–, or pre-200c–transfected Hey cells treated with various chemotherapeutic agents. *Top right,* Trail and FasL; *bottom left,* cisplatin, doxorubicin, and mitomycin C; *bottom right,* paclitaxel, vincristine, and epothilone B. *Asterisks,* statistically significant differences (as determined by Student's *t* tests) between the pre-200c–treated cells versus negative- and mock-transfected controls individually ($P = 5.8 \times 10^{-5}$ and $P = 7.9 \times 10^{-5}$, pre-200c versus mock and negative control, respectively, in the paclitaxel-treated group; $P = 0.0005$ and $P = 0.0009$, pre-200c versus mock and negative control, respectively, in the vincristine-treated group; and $P = 1.3 \times 10^{-5}$ and $P = 2.3 \times 10^{-5}$, pre-200c versus mock and negative control, respectively, in the epothilone B–treated group).

genes significantly different in the pre-200c– versus mock-transfected or negative control–treated cells, but not both, are shown in Fig. 5B. As expected, on addition of miR-200c, E-cadherin (*CDH1*) was up-regulated (on average, 10.8-fold) and *ZEB1* was down-regulated (by 3.1-fold). A complete list of genes significantly affected by miR-200c with fold changes and *P* values is provided

in Supplementary Table S1.[1] Further validation of the microarray results is the fact that 18 putative direct targets of miR-200c, as predicted by bioinformatics using Sanger miRBase, TargetScan, and PicTar databases (including

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[1] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

ZEB1 and *ZEB2*), are differentially regulated in the miR-200c–treated cells (Fig. 5C) as compared with controls. The vast majority (14 of 18) of the direct targets are down-regulated by miR-200c restoration. Many of the genes identified are not predicted miR-200c targets, but are likely downstream of direct targets (as is the case with E-cadherin, being downstream of the direct target, *ZEB1*). Interestingly, a significant number of genes affected by pre-200c treatment were recognized by Ingenuity Pathway Software as belonging to a network of genes involved in cellular migration, and these genes are shown in the cluster depicted in Fig. 5D.

To validate the array data, we performed real-time RT-PCR on *CHK2, ARHGDIB, EPHB1, MAL2, LEPR*, and *ST6GALNAC5* (Fig. 6A) on independent biological samples. These genes represent both predicted direct targets of miR-200c (*LEPR, ST6GALNAC5*) as well as presumed downstream targets (*CHK2, ARHGDIB, EPHB1*, and *MAL2*). We have also confirmed by RT-PCR that *TUBB3* is modestly decreased at the RNA level; however, we observe a more dramatic decrease in TUBB3 protein, indicating that miR-200c may also affect translation of *TUBB3* (Fig. 7A, *left*). To test whether *TUBB3* is a direct target of miR-200c, we cloned the predicted target sequence within the *TUBB3* gene downstream of luciferase in a reporter vector. Figure 7A (*right*) shows that the amount of luciferase is only significantly decreased by the presence of the *TUBB3* 3′-UTR target sequence when miR-200c mimic is transfected into Hec50 cells, and not in the presence of negative control mimic or the mock-transfected cells. In addition, when the target region of the *TUBB3* 3′-UTR was mutated at 3 bp that bind the seed sequence of miR-200c (see *asterisks* in Fig. 7A, *right*), luciferase is no longer reduced, indicating that the miR-200c binding site has been rendered nonfunctional. Furthermore, in the aggressive ovarian cancer cell line Hey, TUBB3 protein levels are even more dramatically reduced by miR-200c (Fig. 7B, *left*) than they are in the Hec50 cells. To assay whether a more pronounced decrease in TUBB3 corresponds with an even greater chemosensitivity to microtubule-targeting agents in the Hey cells, we again performed a cell death ELISA with the panel of chemotherapeutic agents. Similar to the Hec50s, there was no substantial increase in chemosensitivity to agents that cause apoptosis through cell surface receptors or via DNA damage; however, there was a dramatic statistically significant increase in cell death in response to microtubule-targeting agents. The most dramatic increase in response was observed with paclitaxel, in which there is an 82% to 85% increase in chemosensitivity when miR-200c is restored to the cells (Fig. 7B, *bottom right*). There was also a 33% to 35% increase in chemosensitivity to vincristine in the pre-200c–treated cells and a 43% to 50% increase in response to epothilone B. Interestingly, MDA-MB-231 cells have been reported to be resistant to Taxol by a different method (a mutation in class I β-tubulin; ref. 22), and we observe that miR-200c does not restore chemosensitivity to microtubule-targeting agents in this cell line (data not shown).

Discussion

In this report, we examine the effect of restoring miR-200c expression to poorly differentiated, aggressive endometrial, breast, and ovarian cancer cells. Several recent reports have shown the importance of miR-200c, as well as other miR-200 family members, in the regulation of ZEB1 and ZEB2. Because miRNAs commonly function by binding the 3′-UTRs of genes, the conservation of the target sequences in *ZEB1* and *ZEB2* emphasizes the importance of miR-200–mediated regulation of these genes. MiR-200c maintains "epithelial-ness" by suppressing ZEB1 and ZEB2. We show that when we put miR-200c back into aggressive endometrial, breast, and ovarian cancer cells that have lost it (restoring it to levels found in normal epithelial cells), we observe dramatic effects on phenotype and behavior. Restoration of miR-200c causes a nearly complete inhibition of ZEB1 expression and, importantly, a very prominent restoration of E-cadherin protein expression. Others have shown that due to their high degree of sequence homology, other miR-200 family members (miR-141 on the same chromosome, 12p13.31, and miR-200b, miR-200a, and miR-429 on chromosome 1p36.33) have overlapping functions. However, we show that restoration of miR-200c alone is sufficient to suppress ZEB1 expression, restore E-cadherin, reduce invasive capacity, and restore chemosensitivity to microtubule-targeting agents.

Although ZEB1 does repress numerous genes involved in polarity (6, 7), we predicted that such a dramatic reversal of phenotype, invasiveness, and chemosensitivity achieved by restoration of miR-200c could not be solely explained by its ability to target ZEB1 and ZEB2. We suspected that just as miR-200c indirectly maintains E-cadherin expression by directly abolishing ZEB1 (a potent E-cadherin repressor), it also likely both directly and indirectly affects other genes involved in polarity, migratory/invasive capacity, and chemosensitivity. Consequently, we performed expression profiling to identify additional genes statistically significantly affected by restoration of miR-200c in Hec50 endometrial cancer cells.

Whereas it is possible that some direct targets of miR-200c might be missed on a cDNA expression array because miRNAs can affect translation of a target mRNA without causing a change in transcript levels, microarray expression profiling remains a valid method of identifying global gene changes induced by restoration of miR-200c. Indeed, significant alterations in 16 genes (in addition to *ZEB1* and *ZEB2*) predicted by bioinformatics to be direct targets of miR-200c were observed with reintroduction of miR-200c to Hec50 endometrial cancer cells. The majority of the predicted target genes (14 of 18) were down-regulated by miR-200c in our study. Only four (*ANKRD1, ANKRD43, ST6GALNAC5*, and *RAB11FIP1*) were up-regulated. Although most miRNA targets are down-regulated, there is some evidence that miRNAs can target genes for up-regulation by at least two different mechanisms (23, 24). Some of the interesting predicted direct targets significantly down-regulated by miR-200c in our study include other mesenchymal markers, such as fibronectin 1 (*FN1*), which, like ZEB1, is a marker of EMT, and leptin receptor (*LEPR*), which is overexpressed in breast

cancers with poor prognosis and has been implicated in mammary tumorigenesis (25–27). NTRK2 (neurotrophic tyrosine kinase; also known as TrkB) acts as a potent suppressor of anoikis (detachment-induced apoptosis) and is associated with the acquisition of an aggressive tumorigenic and metastatic phenotype *in vivo* (28). QKI, an RNA binding protein, is involved in myelination and binds to mRNAs encoding oncogenes, suggesting a role for QKI in cancer (29, 30).

Like E-cadherin, some of the other genes identified in our screen are known to be repressed by ZEB1; for instance, *MAL2, MARVELD2, PKP3, PPL,* and *TACSTD1* have all previously been identified as ZEB1 targets (6, 7) and were significantly increased by miR-200c in our experiments. *MAL2* is essential for basolateral-to-apical transcytosis and apical trafficking, a defining feature of epithelial cells. Plakophilin 3 (*PKP3*) and periplakin (*PPL*) are components of desmosomes, and *MARVELD2* encodes a tight junction protein. *TACSTD1* (tumor-associated calcium signal transducer 1; also known as EpCAM) encodes a protein expressed on the apical side of epithelial cells.

We also found that restoration of miR-200c increases *ARHGDIB* (Rho GDP-dissociation inhibitor β), an inhibitor of Rho GTPases, which acts as a tumor suppressor and is a negative regulator of migration (reviewed in ref. 31). Thus, in addition to restoring E-cadherin, increasing the tumor suppressor ARHGDIB is a potential mechanism whereby miR-200c reduces the migratory/invasive behavior of Hec50 cells. Other genes involved in cytoskeleton reorganization, such as *WIPF1* (WAS/WASL interacting protein), *EPS8L1* (EPS8-like 1), and *CGN* (cingulin), which regulates tight junctions, may also be involved in the ability of miR-200c to modulate cell motility. EPHB1 (ephrin receptor B1) is a receptor tyrosine kinase involved in cell-cell interactions, angiogenesis, migration, and stem cell polarity (32, 33). In addition, both lysyl oxidase (*LOX*) and fibronectin 1 (*FN1*) are significantly decreased by restoration of miR-200c and they are heavily implicated in metastasis and formation of the premetastatic niche (34–36).

We also show that restoration of miR-200c expression renders Hec50 cells more sensitive to paclitaxel and other microtubule-directed agents such as vincristine and epothilone B, but not to apoptosis-inducing agents that work through death receptors (TRAIL and FasL) or DNA-damaging agents (cisplatin, doxorubicin, or mitomycin C). Intriguingly, our microarray data reveal that the class III β-tubulin (*TUBB3/TUBB4*) gene, a predicted direct target of miR-200c, is significantly down-regulated in the presence of miR-200c. This provided an attractive potential mechanism whereby miR-200c restores chemosensitivity specifically to microtubule-targeting agents because numerous studies have linked overexpression of TUBB3, normally only expressed in neuronal cells, with resistance to microtubule-targeting agents in many types of cancers (37–40). We confirmed its down-regulation in the presence of miR-200c at the RNA level and observed an even more dramatic decrease at the protein level in endometrial and ovarian cancer cells, suggesting that miR-200c may regulate TUBB3 expres-

sion primarily through inhibition of *TUBB3* translation. Furthermore, we show that the predicted miR-200c target site within the *TUBB3* 3′-UTR is indeed a bona fide target of miR-200c, being necessary and sufficient for down-regulation of TUBB3 by miR-200c. In Hey ovarian cancer cells, we observed an even more pronounced decrease in TUBB3 protein in response to restoration of miR-200c than observed in Hec50 endometrial cells, and this corresponds with a dramatic 85% increase in chemosensitivity to paclitaxel. The majority of women with advanced ovarian cancer ultimately relapse with drug-resistant disease with an overall 5-year survival of <50%. Several studies have linked expression of TUBB3 with resistance to microtubule-targeting chemotherapeutics in ovarian cancers (41–44). One of these studies (44) examined the three main mechanisms of paclitaxel resistance (overexpression of *MDR-1* gene, point mutations in α-tubulin and β-tubulin genes, and selective alterations in the expression of β-tubulin isotypes) and found that the only statistically significant difference in the resistant subset of tumors was up-regulation of class III β-tubulin. Thus, we propose that targeting *TUBB3* is a major mechanism whereby miR-200c restores chemosensitivity to microtubule-targeting agents. We propose that in the clinical setting, restoration of miR-200c could render highly aggressive forms of endometrial and ovarian cancers more responsive to microtubule-targeting chemotherapeutic agents and decrease invasive potential as well.

There is increasing evidence that miRNAs play an important role in many aspects of tumorigenesis, and recent reports have implicated the miR-200 family in EMT via direct repression of ZEB1 and ZEB2. Here we extend this developing paradigm by showing that loss of miR-200c is associated with type 2 endometrial carcinomas as well and that restoration of miR-200c to aggressive endometrial cancer cells results in reestablishment of epithelial identity. E-cadherin and other markers of polarity are reexpressed; cells are rendered substantially less invasive and become significantly more sensitive to microtubule-targeting chemotherapeutic agents. We identify additional genes (other than *ZEB1* and *ZEB2*) directly and indirectly targeted by miR-200c, which are likely responsible for restoration of the epithelial state; decrease in migratory capacity; and increased sensitivity to microtubule-targeting chemotherapeutics. We suggest that loss of miR-200c will be predictive of aggressive behavior and resistance to microtubule-targeting agents, and that restoration of miR-200c has potential as a therapeutic strategy for treating highly metastatic, drug-resistant, and thus otherwise relatively untreatable, cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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